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AGRICULTURAL RESEARCH INSTITUTE
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CONTENTS

No. 1

PAGE

Obituary Notice 1

I. The selective fermentation of glucose and fructose by brewer's yeast. By H. Ivekovich 4

II. The chemical investigation of "bios." Part I. By B. T. Narayanan 6

III. The concentration of vitamin B_2. By B. T. Narayanan and J. C. Drummond. (With four figures) 19

IV. The relative vitamin A value of the body and liver oils of certain fish. By B. Ahmad and J. C. Drummond. (With six figures) 27

V. The preservation of the antiscorbutic vitamin in lemon juice. By J. Williams and J. W. Corran 37

VI. The preparation of glutathione from yeast and liver. By N. W. Pirie 51

VII. Determination of volatile substances. By G. Gazenko. (With one figure) 55

VIII. The determination of sucrose by Hanes's modification of the Hagedorn-Jensen method. By E. H. Callow. (With one figure) 57

IX. Studies on hemicelluloses. I. The hemicelluloses of wheat bran. By F. W. Norris and I. A. Preece 59

X. The solubility of glycogen. By M. Kerly. (With four figures) . . 67

XI. Further evidence for a third accessory "B" factor. By V. Reader. (With two figures) 77

XII. Uricase and its action. By S. J. Przylecki 81

XIII. The antiscorbutic potency of apples. By M. F. Bracewell, E. Hoyle and S. S. Zilva 82

XIV. Observations upon the reducing substances of pigeons' blood. By J. M. Gulland and R. A. Peters. (With one figure) 91

XV. Heat-stability of the (anti-dermatitis, "anti-pellagra") water-soluble vitamin B_2. By H. Chick and M. H. Roscoe 105

XVI. Sphingomyelin, phrenosin and kerasin in films one molecule thick on water. By K. Turner and M. M. Watson. (With one figure) . . 113

XVII. The phosphoric esters of alcoholic fermentation. By R. Robison and W. T. J Morgan 119

XVIII. The colour reactions and absorption spectra of sterols in relation to structure. By I. M. Heilbron and F. S. Spring 133

XIX. Absorption spectra in relation to vitamin A. By R. A. Morton, I. M. Heilbron and F. S. Spring. (With two figures) 136

XX. The inhibition of lactic acid formation in cancer and muscle. By S. T. Harrison and E. Mellanby 141

XXI. The denaturation of proteins. VI. The titration of the basic and acidic groups in egg-albumin. By N. Booth. (With three figures) . . 158

XXII. Lactic acid formation in muscle extracts. V. A comparison between soluble starch and glycogen in respect of lactic acid formation and phosphoric ester accumulation. By D. Stiven 169

PAGE

XXIII. Lactic acid formation in muscle extracts. VI. The influence of irradiation on lactic acid formation and phosphoric ester accumulation from glycogen. By D. STIVEN. (With two figures) 172

XXIV. Structure and enzyme reactions. VIII. The physical state of the sorbent. By S. J. PRZYLECKI and M. GURFINKEL 179

XXV. The stereochemical specificity of esterases. I. The affinity of liver esterases for optically active alcohols. By D. R. P. MURRAY and C. G. KING. (With six figures) 190

XXVI. The vitamin B content of grass seeds in relationship to manures. By M. J. ROWLANDS and B. WILKINSON. (With eleven figures) 199

XXVII. Note upon the sulphur linkage in wool. By C. RIMINGTON . 205

XXVIII. A quantitative study of succinic acid in muscle. III. Glutamic and aspartic acids as precursors. By D. M. NEEDHAM. (With four figures) . 208

No. 2

OBITUARY NOTICE 229

XXIX. The effect of cereal extracts on blood-calcium. By L. MIRVISH . 233

XXX. The tyrosinase-tyrosine reaction. VII. The action of tyrosinase on certain substances related to tyrosine. By W. L. DULIÈRE and H. S. RAPER. (With one figure) 239

XXXI. Sulphur in organic and inorganic combination in pasture grasses. By H. A. A. AITKEN 250

XXXII. Surface films of batyl, chimyl and selachyl alcohols. By B. C. J. G. KNIGHT. (With two figures) 257

XXXIII. The decarboxylation of pectin. By F. V. LINGGOOD . . 262

XXXIV. The composition of the mixed fatty acids present in the glycerides of cod-liver and certain other fish-liver oils. By K. D. GUHA, T. P. HILDITCH and J. A. LOVERN 266

XXXV. The effect of glutathione on the determination of blood-sugar. By F. K. HERBERT, M. C. BOURNE and J. GROEN. (With one figure) . . 291

XXXVI. The non-sugar reducing substances of human blood, with special reference to glutathione. By F. K. HERBERT and M. C. BOURNE . 299

XXXVII. On the catalysis of the oxidation of cysteine and thioglycollic acid by iron and copper. By K. A. C. ELLIOTT. (With fourteen figures) . 310

XXXVIII. A study of the composition of human milk in the later periods of lactation and a comparison with that of early milk. By S. T. WIDDOWS, M. F. LOWENFELD, M. BOND and E. I. TAYLOR 327

XXXIX. Fermentation by yeast preparations. By A. HARDEN and M. G. MACFARLANE. (With two figures) 343

XL. Phosphoric esters in alcoholic fermentation. II. Pyrophosphate in yeast preparations. By E. BOYLAND. (With one figure) 350

XLI. Note on the determination of total calcium in blood-serum. By J. PATTERSON 355

XLII. Studies on gelatin. XI. Diamino-nitrogen of gelatin. By K. V. THIMANN. (With three figures) 357

XLIII. The precipitation of the basic amino-acids of proteins with phosphotungstic acid. By K. V. THIMANN. (With Plate I and three figures) . 368

XLIV. Investigations on the hydrolysis products of fish muscle. I. The diamino-nitrogen of cod muscle. By J. C. KERNOT, J. KNAGGS and N. E. SPEER 379

PAGE

XLV. The influence of the hypophysis on metabolism, growth and sexual organs of male rats and rabbits. II. Influence of extracts of hypophysis on the body weight, weight of fat, of sexual organs and of endocrine organs of rats. By V. Korenchevsky 383

XLVI. The Hagedorn-Jensen method applied to various sugars. Relation of reducing power to configuration. By H. Sobotka and M. Reiner. (With one figure) 394

XLVII. A note on the oxygen consumption of nerve in the presence of glucose and galactose. By M. Sherif and E. G. Holmes. (With two figures) 400

XLVIII. Studies on pectin. IV. The oxidation of pectin by Fenton's reagent and its bearing on the genesis of the hemicelluloses. By A. G. Norman and [illegible]. W. Norris 402

XLIX. Xanthine oxidase in the avian embryo. By E. J. Morgan . . 410

L. Factors affecting the catalytic action of copper in the oxidation of cysteine. By C. A. Elvehjem. (With eleven figures) 415

LI. The action of salts on fumarase. I. By P. J. G. Mann and B. Woolf. (With six figures) 427

LII. The chemistry of oestrin. III. An improved method of preparation and the isolation of crystalline material. By G. F. Marrian. (With Plate II) 435

LIII. Studies on antiglyoxalase. I. The action of pancreatic extract on phenylglyoxal. By J. O. Giršavičius. (With two figures) 446

LIV. The alleged contamination of carotene by vitamin A. By N. S. Capper. (With one figure) 453

LV. The isolation of *l*-thyroxine from the thyroid gland by the action of proteolytic enzymes. By C. R. Harington and W. T. Salter 456

LVI. The properties of pure glutathione. By N. U. Meldrum and M. Dixon. (With twenty-three figures) 472

LVII. Further experiments on cancer-producing substances. By E. L. Kennaway 497

LVIII. The spectra of cancer-producing tars and oils and of related substances. By I. Hieger. (With Plates III and IV) 505

LIX. Oxidation-reduction potentials of cultures of haemolytic streptococci. I. By L. F. Hewitt. (With eleven figures) 512

LX. Studies in gas production by bacteria. I. Apparatus for the measurement of the rate of gas production. By J. A. Cranston. (With three figures) 525

LXI. Studies in gas production by bacteria. II. Denitrification and bacterial growth phases. By B. Lloyd and J. A. Cranston. (With ten figures) 529

LXII. The production of hydrogen sulphide by animal tissues. By E. Sluiter. (With seven figures) 549

LXIII. A redetermination of the titration dissociation constants of arginine and histidine with a demonstration of the zwitterion constitution of these molecules. By T. W. Birch and L. J. Harris. (With two figures) . 564

No. 3

Obituary Notices 577

LXIV. The reduction of methylene blue in milk. The influence of light. By H. R. Whitehead 579

LXV. The traces of combustible gases in human expired air. By T. R. Parsons 585

PAGE

LXVI. The influence of arginine upon the growth rate of a transplantable tumour in the mouse. By E. Gilroy 589

LXVII. A study of the acid-base equilibrium of the ocular humours. By P. W. Salit. (With one figure) 596

LXVIII. The inactivation of trypsin by heat. By J. Pace. (With one figure) 606

LXIX. Insulin and increase in weight of young animals. By J. J. R. Macleod, H. E. Magee and W. Middleton 615

LXX. The characteristic absorption of ultra-violet radiation by certain purines. By E. R. Holiday. (With two figures) 619

LXXI. The isolation of histamine from the heart. By W. V. Thorpe . 626

LXXII. The absorption and excretion of calcium and phosphorus by rats receiving excessive doses of irradiated ergosterol By E. Watchorn. (With two figures) 631

LXXIII. The chemical aspect of the drying of timber. By W. G. Campbell and J. Booth 641

LXXIV. Studies on pectin. V. The hydrolysis of pectin. By A. G. Norman and J. T. Martin 649

LXXV. The phosphorus distribution in blood and the calcium and phosphorus excretion during hypervitaminosis D. By C. A. Ashford. (With three figures) 661

LXXVI. Oxidation-reduction potentials of cultures of *C. diphtheriae*. I. By L. F. Hewitt. (With six figures) 669

LXXVII. Oxidation-reduction potentials of staphylococcal cultures. I. By L. F. Hewitt. (With five figures) 676

LXXVIII. The fat-soluble vitamins of milk. By M. E. F. Crawford, J. Golding, E. O. V. Perry and S. S. Zilva. (With seven figures) . . 682

LXXIX. Vitamin A and carotene. V. The absence of the liver oil vitamin A from carotene. VI. The conversion of carotene to vitamin A *in vivo*. By T. Moore. (With two figures) 692

LXXX. Phosphoric esters in alcoholic fermentation. III. The lag between phosphate esterification and carbon dioxide evolution. IV. Oxidation-reduction potentials of yeast-preparations. By E. Boyland. (With one figure) 703

LXXXI. Carbohydrate metabolism in birds. II. Brain localisation of lactic acidosis in avitaminosis B, and its relation to the origin of symptoms. By H. W. Kinnersley and R. A. Peters 711

LXXXII. The metabolism of developing salmon eggs. I. The significance of hatching and the rôle of water in development. By F. R. Hayes. (With two figures) 723

LXXXIII. The metabolism of developing salmon eggs. II. Chemical changes during development. By F. R. Hayes. (With four figures) . . 735

LXXXIV. The micro-determination of hydroxyl groups. By P. M. Marrian and G. F. Marrian. (With one figure) 746

LXXXV. Natural anthocyanin pigments. I. The magenta flower pigment of *Antirrhinum majus*. By R. Scott-Moncrieff. (With six figures) . 753

LXXXVI. Natural anthocyanin pigments. II. The magenta flower pigment of *Primula polyanthus*. By R. Scott-Moncrieff. (With three figures) 767

LXXXVII. The influence of insulin on the distribution of glycogen in normal animals. By B. Corkill 779

LXXXVIII. The comparative rates of absorption of sugars from the human intestine. By R. A. McCance and K. Madders. (With five figures) 795

PAGE

LXXXIX. Studies on the kinetics of haemolytic systems. IV. A further study of the series of Ryvosh. By E. PONDER, G. SASLOW and J. F. YEAGER 805

XC. The determination of the quantity of free water in erythrocytes. By C. KREVISKY 815

XCI. The use of the Barcroft apparatus for the measurement of tissue respiration. By M. DIXON and K. A. C. ELLIOTT. (With ten figures) . . 820

XCII. The oxygen capacity of the blood in one hundred cases of mental disorder. By D. L. WOODHOUSE and F. A. PICKWORTH. (With three figures) 834

No. 4

XCIII. The nature of the blood-sugar. By L. B. WINTER . . . 851

XCIV. The dissociation of calcium citrate. By W. J. WILEY. (With two figures) 856

XCV. Observations on a diatom (*Nitzschia closterium* W. Sm.) as a source of vitamin A. By B. AHMAD. (With three figures) 860

XCVI. The composition of the fatty acids present as glycerides in the liver oil of the thresher shark (*Alopoecia vulpes*). By J. A. LOVERN . . 866

XCVII. Vitamin A of butter. By R. A. MORTON and I. M. HEILBRON. (With three figures) 870

XCVIII. The action of the cinchona and certain other alkaloids in bird malaria. By J. A. GOODSON, T. A. HENRY and J. W. S. MACFIE . . . 874

XCIX. The inactivation of pancreatic lipase by heat. By I. H. MCGILLIVRAY. (With three figures) 891

C. Carbohydrate metabolism of normal and tumour tissue. Part I. A method for the measurement of the respiratory quotient. By F. DICKENS and F. ŠIMER. (With two figures) 905

CI. Oxidations in central and peripheral nervous tissue. By E. G. HOLMES. (With seven figures) 914

CII. Selective fermentation. I. Alcoholic fermentation of glucose, fructose and mannose mixtures. By H. SOBOTKA and M. REINER 926

CIII. The heat-stability of the (antidermatitis, "anti-pellagra") water-soluble vitamin B_2. II. By H. CHICK and A. M. COPPING 932

CIV. The stability of watery solutions of the oxytocic principle of the pituitary gland. By J. H. GADDUM. (With two figures) 939

CV. Synthesis of the *i*-hydroxy-asparagines. By A. C. CHIBNALL. A note on their dissociation constants. By R. K. CANNAN 945

CVI. The effect of cryptorchidism and of castration on the chemical composition of rats. By V. KORENCHEVSKY and M. DENNISON 954

CVII. An improved design of the Van Slyke apparatus for the estimation of amino-nitrogen. By S. J. FOLLEY. (With two figures) 961

CVIII. A sensitive reaction for cysteine. By R. FLEMING 965

CIX. Vitamin C and the rat's diet. By G. A. HARTWELL. (With one figure) 967

CX. Studies on hemicelluloses. II. The hemicelluloses of maize cobs. By I. A. PREECE 973

CXI. Carotene and vitamin A. The transformation of carotene into vitamin A as shown by a study of the absorption spectra of rat-liver oils. By N. S. CAPPER. (With one figure) 980

CXII. Note on the possible mechanism of diphtheria toxoid formation. By L. F. HEWITT. (With two figures) 893

PAGE

CXIII. The specific refraction increments of serum-albumin and serum-globulin. By G. S. Adair and M. E. Robinson. (With one figure) . . 993

CXIV. The action of pepsin on gelatin. By R. K. Cannan and E. Muntwyler. (With two figures) 1012

CXV. The chemistry of oestrin. IV. The chemical nature of crystalline preparations. By G. F. Marrian 1021

CXVI. Properties of the comb-growth-promoting substance obtained from testes and urine. By E. C. Dodds, A. Greenwood, H. Allan and E. J. Gallimore. (With three figures) 1031

CXVII. On the mechanism of sugar excretion. I. Glucose. By T.-G. Ni and P. B. Rehberg. (With three figures) 1039

CXVIII. Note on Montignie's isomer of cholesterol, and the effect ot heat on cholesterol. By Y. Kaishio and J. A. Gardner 1047

CXIX. The action of arsenate on hexosephosphatase. By M. G. Macfarlane 1051

CXX. Chemical change in gelatin resulting from the method of storage. By K. V. Thimann and A. B. Page. (With one figure) 1058

CXXI. The effects of cystine diet on keratin composition in rabbit wool. By J. Barritt, A. T. King and J. N. Pickard 1061

CXXII. Oxidation-reduction studies in relation to bacterial growth. I. The oxidation-reduction potential of sterile meat broth. By B. C. J. G. Knight. (With two figures) 1066

CXXIII. Oxidation-reduction studies in relation to bacterial growth. II. A method of poising the oxidation-reduction potential of bacteriological culture media. By B. C. J. G. Knight. (With two figures) 1075

CXXIV. Zwitterions. I. Proof of the zwitterion constitution of the amino-acid molecule. By L. J. Harris. II. Amino-acids, polypeptides, etc., and proteins as zwitterions, with instances of non-zwitterion ampholytes. By T. W. Birch and L. J. Harris. (With eighteen figures) 1080

CXXV. Variations in the component fatty acids of butter due to changes in seasonal and feeding conditions. By T. P. Hilditch and J. J. Sleightholme 1098

CXXVI. The colorimetric determination of cystine by means of the uric acid reagent. By C. Rimington 1114

CXXVII. Lactic acid oxidation in brain with reference to the "Meyerhof cycle." By E. G. Holmes and C. A. Ashford. (With one figure) . . . 1119

CXXVIII. The rate of liberation of arginine in tryptic digestion. By J. A. Dauphinee and A. Hunter. (With three figures) 1128

CXXIX. The determination of blood-sugar. I. Critical analysis of the reduction of alkaline copper reagents by glucose and other substances. By S. L. Tompsett 1148

CXXX. The determination of blood-sugar. II. The effect of different deproteinising agents upon the estimation of blood-sugar. By S. L. Tompsett 1164

CXXXI. Some factors involved in the experimental production of pellagra in rats. I. Preliminary. By V. R. Leader. (With three figures) . . 1172

CXXXII. Comparison of the effects of arginine and thyroxine upon tumour growth-rate in the mouse. By E. Gilroy. (With five figures) . . 1181

CXXXIII. A new method for the separation of the products of protein hydrolysis. By M. A. B. Brazier 1188

CXXXIV. The action of insulin on the glycogen distribution in normal animals. By M. W. Goldblatt 1199

CXXXV. Studies in photosynthesis. I. The formaldehyde hypothesis. By E. C. Barton-Wright and M. C. Pratt 1210

PAGE

CXXXVI. Studies in photosynthesis. II. The first sugar of carbon assimilation and the nature of the carbohydrates in the narcissus leaf. By E. C. Barton-Wright and M. C. Pratt. (With seven figures) . . . 1217

CXXXVII. The chemistry of the white rots of wood. I. The effect on wood substance of *Polystictus versicolor* (Linn.) Fr. By W. G. Campbell . 1235

CXXXVIII. The disturbance of metabolism produced by bony and non-bony injury, with notes on certain abnormal conditions of bone. By D. P. Cuthbertson 1245

No. 5

CXXXIX. Lipase and its action. I. The synthetic action of pancreatic lipase in the system: oleic acid-glycerol-water-dissolved lipase. By E. A. Sym. (With two figures) 1265

CXL. On the invertase-accelerator of serum, with observations on other enzyme-accelerators reported in serum. By J. Fine. (With five figures) . 1282

CXLI. Amino-acids of tissues. IV. The diamino-acid content of muscle tissue of different classes of animals. By J. L. Rosedale and J. P. Morris 1294

CXLII. Amino-acids of tissues. V. Monoamino-acids of the horse mackerel (*Caranx rottleri*). By J. L. Rosedale and C. J. Oliveiro . . 1297

CXLIII. The metabolism of normal and tumour tissue. II. The respiratory quotient, and the relationship of respiration to glycolysis. By F. Dickens and F. Simer. (With Plate V) 1301

CXLIV. The fatty acids in the liver of the sheep. By K. Turner . . 1327

CXLV. The enzymic synthesis of raffinose. By A. V. Blagoveschenski 1337

CXLVI. Uricase and its action. II. Bacterial nature of the action of uricolytic extracts and dialysates. By R. Truszkowski 1340

CXLVII. Uricase and its action. III. Comparison with activated charcoal. Contact nature of the action of uricase. By R. Truszkowski. (With two figures) 1349

CXLVIII. Uricase and its action. IV. Preparation and properties of ox-kidney uricase. By R. Truszkowski. (With two figures) . . . 1359

CXLIX. The urocarmine reaction. By W. R. Fearon and A. G. Thompson 1371

CL. The effect of freezing in a concentrated solution of sodium chloride on the colour of red muscle. By J. Brooks. (With two figures) . . . 1379

CLI. Avitaminosis B in relation to tumour growth. By E. Gilroy . 1384

CLII. Studies on the metabolism of animals on a carbohydrate-free diet. I. The distribution of glycogen and fat in the liver of animals fed on a carbohydrate-free diet. By A. Hynd and D. L. Rotter 1390

CLIII. A study of the bile pigments in relation to the Van den Bergh reaction. By W. J. Griffiths and G. Kaye 1400

CLIV. The glycogen of mammalian muscle and its behaviour after death. By I. A. Anderson and J. J. R. Macleod 1408

CLV. The behaviour of glutathione in yeast. By N. U. Meldrum . . 1421

CLVI. The determination of cellulose in straws. By S. H. Jenkins . 1428

CLVII. The biochemistry of aluminium. I. Excretion and absorption of aluminium in the pig. By K. Mackenzie 1433

CLVIII. The mode of action of tyrosinase. By C. E. M. Pugh . . 1442

CLIX. An improved method for the determination of iodine in blood. By H. A. A. Aitken. (With one figure) 1456

PAGE

CLX. The absorption of water by gelatin. IV. The influence of temperature. By D. Jordan-Lloyd. (With six figures) 1460

CLXI. The absorption of water by gelatin. V. The influence of calcium hydroxide. By W. B. Pleass. (With three figures) 1472

CLXII. The vitamin B_2 content of cereals and the supposed connection between human pellagra and deficiency of this vitamin. By W. R. Aykroyd 1479

CLXIII. The alleged presence of bile salts in normal blood. By E. Walker 1489

CLXIV. The chemistry of the products of *Cocos nucifera*. I. By J. P. C. Chandrasena 1493

CLXV. Oxidation-reduction studies in relation to bacterial growth. III. The positive limit of oxidation-reduction potential required for the germination of *B. tetani* spores *in vitro*. By B. C. J. G. Knight and P. Fildes. (With three figures) 1496

CLXVI. The question of the identity of a bacterial growth-promoting factor with vitamin B_1. By J. G. Davis and J. Golding. (With one figure) 1503

CLXVII. The preparation and properties of the gonad-stimulating hormone from the urine of pregnancy. By F. Dickens. (With Plate VI) . . 1507

CLXVIII. Pyruvic acid in bacterial metabolism. With an account of the methods used for the detection and determination of pyruvic acid. By R. P. Cook. (With one figure) 1526

CLXIX. A comparison of the dehydrogenations produced by *B. coli communis* in the presence of oxygen and methylene blue. By R. P. Cook. (With nine figures) 1538

CLXX. Oxidation-reduction potentials of Pneumococcus cultures. I. By L. F. Hewitt. (With six figures) 1551

CLXXI. A cell for the measurement of the specific conductivity of the blood-serum. By W. R. Ashby. (With one diagram) 1557

CLXXII. Irradiated ergosterol and calcium-free diet: effect on calcium and phosphorus metabolism. By E. Watchorn 1560

CLXXIII. Production of ammonia by surviving kidney tissue. II. Studies on the possible precursors of urinary ammonia. By B. E. Holmes and A. Patey. (With two figures) 1564

CLXXIV. Manganese in relation to nutrition. By M. B. Richards . 1572

No. 6

CLXXV. "Diet 4" for breeding rats for work on vitamin A. By S. V. Gudjónsson. (With six figures) 1591

CLXXVI. The influence of foodstuffs on the acid-base balance of cattle urine. By F. J. Warth and N. K. Ayyar. (With one figure) . . . 1595

CLXXVII. The colorimetric determination of the tyrosine and tryptophan content of various crude protein concentrates. By W. D. McFarlane and H. L. Fulmer 1601

CLXXVIII. Studies in embryonic mortality in the chick. I. The effect of diet upon the nitrogen, amino-nitrogen, tyrosine, tryptophan, cystine and iron content of the proteins and on the total copper of the hen's egg. By W. D. McFarlane, H. L. Fulmer and T. H. Jukes. (With one figure) . 1611

CLXXIX. Vitamin B deficiency in the rat. Bradycardia as a distinctive feature. By A. N. Drury, L. J. Harris and C. Maudsley. (With four figures) 1632

PAGE

CLXXX. Vegetable proteins. I. The proteins of *Dolichos lab lab*. By D. NARAYANAMURTI and C. V. RAMASWAMI. (With one figure) 1650

CLXXXI. Irradiation of *Dolichos* tyrosinase. By D. NARAYANAMURTI and C. V. RAMASWAMI 1655

CLXXXII. Further observations upon the correlation between the arginine and vitamin B content of diets, and the effect of thyroxine upon tumour growth. By E. GILROY. (With two figures) 1659

CLXXXIII. On the organic matter given off by algae. By A. KROGH and E. LANGE with W. SMITH 1666

CLXXXIV. The behaviour of δ-keto-*n*-hexoic acid in the perfused liver. By W. L. DULIÈRE and H. S. RAPER. (With one figure) 1672

CLXXXV. The male hormone. IV. By C. FUNK and B. HARROW . 1678

CLXXXVI. Purinolytic enzymes of the human organism. By R. TRUSZKOWSKI 1681

CLXXXVII. The antiscorbutic fraction of lemon juice. IX. By S. S. ZILVA. (With two figures) 1687

CLXXXVIII. The denaturation of proteins. VII. Denaturation in the presence of alcohol. By N. BOOTH. (With one figure) 1699

CLXXXIX. The effect of light and salts on gelatin. By A. GALINSKY . 1706

CXC. On the presence of a volatile organic chlorine compound in blood. By N. MORRIS and S. MORRIS. (With three figures) 1716

CXCI. The action of arsenite on tissue respiration. By A. SZENT-GYÖRGYI 1723

CXCII. The effect of cooking on the digestibility of meat. By W. M. CLIFFORD. (With one figure) 1728

CXCIII. Determination of maltose in plant extracts by maltase. By N. NARASIMHAMURTY and M. SREENIVASAYA 1734

CXCIV. The correlation of the oxidation of certain phenols and of dimethyl-*p*-phenylenediamine by bacterial suspensions. By F. C. HAPPOLD . 1737

CXCV. The alcohol-solubility of the anti-dermatitis, more heat-stable vitamin B_2 constituent of the vitamin B complex. By H. CHICK and A. M. COPPING 1744

CXCVI. Egg-white as a source of the anti-dermatitis vitamin B_2. By H. CHICK, A. M. COPPING and M. H. ROSCOE 1748

CXCVII. The distribution of the vitamin B complex. I. Leafy vegetables. By M. H. ROSCOE 1754

CXCVIII. The composite nature of the water-soluble vitamin B. III. Dietary factors in addition to the anti-neuritic vitamin B_1 and the anti-dermatitis vitamin B_2. By H. CHICK and A. M. COPPING. (With one figure) 1764

CXCIX. The biological values of proteins. I. A method for measuring the nitrogenous exchange of rats for the purpose of determining the biological value of proteins. By H. CHICK and M. H. ROSCOE 1780

CC. Selective fermentation. II. Fermentation of sugar mixtures by Sauterne yeast. By H. SOBOTKA and M. REINER 1783

CCI. The non-sugar reducing substances of human blood in pathological conditions. By F. K. HERBERT and M. C. BOURNE. (With one figure) . . 1787

CCII. The biological values of proteins. II. The biological value of purified caseinogen and the influence of vitamin B_2 upon biological values, determined by the balance sheet method. By M. A. B. FIXSEN. (With one figure) 1794

CCIII. A comparison of the colorimetric and electrometric methods for the determination of the p_H of serum or plasma. By R. J. FOSBINDER and J. W. SCHOONOVER 1805

PAGE

CCIV. Heart block in pigeons, curative factor. By C. W. Carter . . 1811

CCV. A quantitative comparison of the curative activity of torulin (vitamin B_1) upon the adult pigeon and the adult white rat. By H. W. Kinnersley, R. A. Peters and V. Reader 1820

CCVI. The curative activity of the antineuritic vitamin of rice. By B. C. P. Jansen, H. W. Kinnersley, R. A. Peters and V. Reader . . 1824

CCVII. The assay of vitamin B_4. By V. Reader. (With two figures) . 1827

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CCXVIII. The reaction of antimony trichloride with cod-liver oil and its unsaponifiable fraction. By E. L. Smith and V. Hazley. (With three figures) 1942

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Index 1967

OBITUARY NOTICE.

THOMAS BARLOW WOOD (1869–1929).

Thomas Barlow Wood, Professor of Agriculture in the University of Cambridge, was born the son of a farmer in 1869 at Field Dalling, Holt, Norfolk: he was educated at the Newcastle High School, Staffordshire and at Caius College, Cambridge, where he took chemistry as his main subject. University Extension work was then very much to the fore and, like other promising graduates of the time, he thought of it as a good start for a career. After taking the Tripos he accordingly attended in July, 1891, the newly established short course in agricultural chemistry held by Henry Robinson, assistant to Prof. Liveing, the first instruction in agricultural science ever given in Cambridge.

He was then sent to Devon by the University Extension Syndicate to lecture on Soils and Manures, but he left at the end of the year to return in January 1892 to Norfolk, where he occupied substantially the position of a modern county organiser. He taught science in the County School during the week, taught elementary teachers on Saturdays, lectured to farmers at the various country towns during winter, and conducted field experiments for the Norfolk Chamber of Agriculture during the rest of his time. It was a hard apprenticeship, but it gave him a knowledge of farming and an insight into the farmer's character which stood him in good stead throughout his life. Two years he remained here: then in January 1894 he returned to Cambridge at the request of Prof. Liveing to take the place of Robinson who had recently died. Agricultural chemistry was just gaining recognition and it is greatly to Liveing's credit that he was one of the first to see its importance. Wood's duties included lectures and demonstrations to students and field demonstrations to farmers; he also found time for research work on Indian hemp and cannabinol, done in part with the ill-fated Spivey who was burnt to death through the breaking of a flask containing carbon disulphide. But the funds were precarious and the whole scheme might have collapsed had not Marshall Ward and Austin Keen, Education Secretary of the Cambridge County Council, vigorously supported it. Ten neighbouring counties were induced to contribute, but at a price. Wood had to conduct experiments in all of them and his life was a strenuous round of lecturing and demonstrating in the mornings, visits to farms in the afternoons—often prolonged so that he returned too late for Hall—and then close attention to correspondence in the evenings. He bore the burden cheerfully, and recognition came in the form of more funds: finally in 1899 the Drapers' Company endowed the Chair of Agriculture.

It was a disappointment to Wood that he was not elected Professor but was given only a Lectureship. The idea was then held that a Professor of Agriculture should not be a trained scientific investigator but a technical man, and both the first and second holders of the Chair were chosen on these grounds. Wood, however, gave them ungrudging and loyal support throughout the seven years of their tenure of office; he never let his personal feelings count. In 1907, the Chair being again vacant, Wood was appointed Professor and from that time both he and the School of Agriculture developed at an amazing rate. The beautiful and effective building was completed (the scheme had been well started by T. H. Middleton), a second Professorship and several Lectureships and Readerships were set up, the farm was put on a sound basis, and the educational arrangements were completely overhauled and very much strengthened. Holidays snatched from work were spent on his father's farm at Holt, which he afterwards took over and ran himself. Close contact was kept with farmers and recognised agricultural organisations. All the time, year in and year out, amidst all this strenuous labour, there was kept going in an atmosphere of peace and quietness a steady development of research work, beginning in a small way, but growing constantly until now the Cambridge School is one of the great centres of agricultural research in the world.

The work had developed gradually but continuously, and Wood took on first one and then another activity till finally the load was more than any one man could bear. There were some uncomfortable warnings, and his friends had been anxious about his health for several years past, but he continued bravely with his work till a few months before he died.

Wood has two great claims to be remembered.

As a teacher he was admirable, stimulating and vigorous, especially in conversation with his pupils. He had a quick and sympathetic eye for a student's difficulty, a ready flow of racy English—for much of which he was indebted, as he once told me, to the classical master at school with whom he read Horace—and an abounding kindliness and genial hospitality. His colleagues and students all knew they could count upon his help whenever it was needed. His students are now found all over the world occupying some of the most important positions in agriculture.

As a research worker he began with soils and crop production, the natural way into the subject, but soon turned over to animal nutrition, always an important matter to the Norfolk farmer, who spends large sums of money in growing and buying food-stuffs for the farm animals. His first important papers were on the composition of the mangold crops and on the causes of the strength of wheat, which opened the way to much subsequent work on gluten. When after the war he settled down to work in the new buildings he organised investigations on the digestibility of various crops, including oat and tare silage and grass (this latter being studied by H. E. Woodman), and he encouraged Capstick to build the calorimeter by use of which he carried out important studies of the energy changes in the growing pig. Finally, in 1928, he summarised his long experience in a paper that bids fair to become classical: "The scientific basis of rationing animals[1]." Starting with the equation he had already established:

$$R = Am + gc$$

where R is the ration per unit time in terms of net energy[2], either calories or lb. of starch equivalent:

A = surface area of the animal:
m = maintenance requirement per square metre per unit time:
g = live weight increase in lb. per unit time:
c = calories or lb. of starch equivalent required to give 1 lb. increase in live weight:

he showed how this could be used for the rationing of fatting sheep and the production of milk. He had long tried, but without success, to apply it to the production of work: unhappily he was never to finish this problem: the difficulty is to measure the quantity of movement of the animal.

These lines of research were vigorously taken up by an enthusiastic band of workers who it is hoped will continue to develop the subject. Just as the School of Agriculture is the most fitting visible memorial to Wood, so the tradition he created of research in animal nutrition seems likely to remain as his most enduring scientific memorial.

E. J. RUSSELL.

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[1] *J. Agric. Sci.* (1928), **18**, 486, jointly with J. W. Capstick.

[2] Net energy is the energy an animal can use for physiological purposes, *i.e.* the energy left after deducting whatever is necessary for the digestion of the food.

I. THE SELECTIVE FERMENTATION OF GLUCOSE AND FRUCTOSE BY BREWER'S YEAST.

By H. IVEKOVICH.

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(*Received November 26th, 1929.*)

Hopkins [1928] has published a paper concerning the selective fermentation of a mixture containing equal quantities of glucose and fructose. This paper gives valuable information on the fermentation phenomena of solutions containing those two sugars. In the present note we shall deal only with that part of this interesting paper, which treats of the ratio of the fermentation rates of the two sugars present.

The ratio of the two specific rates was calculated by Hopkins by means of the formula:

$$\frac{k_g}{k_f} = \frac{1/t \ln a_g/(a_g - x_g)}{1/t \ln a_f/(a_f - x_f)} = \frac{\ln a_g - \ln (a_g - x_g)}{\ln a_f - \ln (a_f - x_f)} = K,$$

where a_g is the initial concentration of glucose, a_f the initial concentration of fructose, $(a_g - x_g)$ the concentration of glucose at time t, $(a_f - x_f)$ the concentration of fructose at time t.

The constant K which, as it appears from this formula, is the quotient of k_g and k_f, *i.e.* of the constants of the fermentation reactions of glucose and fructose, assuming that they are monomolecular reactions, varies from 1·68 to 1·88, its mean value being 1·78. The value of this constant does not appear to vary much beyond the limits of experimental errors.

It is interesting to note that we have found the same value for this constant by an entirely different way, which we described in a paper [1927] which has escaped the attention of Hopkins. Studying the influence of animal charcoal on fermentation by yeast and the application of charcoal in the polarimetric analysis of fermented solutions of sugars we observed the fact that the (negative) rotation of a solution containing equal quantities of glucose and fructose increases up to a maximum, and decreases continually beyond that maximum.

This maximum of laevo-rotation is a very important point, as it throws some light upon the ratio of the disappearance rates of the two sugars from the solution. At the moment of the maximum rotation the ratio of the fermentation rates of the two sugars is given by the ratio of their specific rotations, *i.e.* by the quotient 93·0/52·5 = 1·78.

For each 1·78 mol. of glucose, 1 mol. of fructose disappears. In fact, if the glucose fermented at a faster rate than is given by this ratio, the rotation could not decrease after that moment of maximum; if, on the other hand, it fermented at a slower rate, it could not possibly increase before that moment, as the observed rotation is but the sum of the negative and positive rotations of the fructose and the glucose respectively in the solution.

In the paper quoted we stressed the fact that the ratio of the disappearing sugars in the solution was 1 : 1·78, *i.e.* this figure gives the ratio of fermentation rates of sugars at the moment at which the curve reaches its maximum. The fact that this figure coincides to the second decimal with Hopkins's mean value of the constant K must be regarded as accidental.

The constants k_g and k_f of the fermentation reactions are the quotients of the reaction rates and the quantity of substance left in the solution at the time t. The constant K is in consequence

$$K = \frac{k_g}{k_f} = \frac{c_g/q_g}{c_f/q_f} = 1{\cdot}78,$$

where c_g and c_f are the fermentation rates of glucose and fructose respectively, and q_g and q_f the respective quantities of glucose and fructose left in the solution at the time t.

If $q_g = q_f$, *i.e.* if we have a solution of equal quantities of glucose and fructose, K will be

$$K = \frac{c_g}{c_f} 1{\cdot}78,$$

i.e. glucose will ferment 1·78 times faster than fructose.

By means of this formula, the ratio of the fermentation rates of glucose and fructose may be calculated for any concentration of the two sugars. The figure 1·78, which we found as quotient of the specific deviations of fructose and glucose, is but the extreme case of Hopkins's general formulation.

For a solution containing glucose and fructose in proportion of 1·78 : 1 the ratio of the rates of fermentation, all other things being equal, will be 3·1 : 1, as

$$\frac{c_g/1{\cdot}78}{c_f/1} = 1{\cdot}78, \text{ where } c_f = 1.$$

Of course, this is true only for the conditions under which Hopkins's equation $K = \frac{k_g}{k_f} = 1{\cdot}78$ also holds.

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II. THE CHEMICAL INVESTIGATION OF "BIOS."

PART I.

BY BINDIGANAVALE TIRUMALACHAR NARAYANAN.

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(*Received November 27th, 1929.*)

INTRODUCTION.

FEW scientific papers have given rise to more controversy than that in which Wildiers [1901] described "bios." After nearly thirty years of investigation, a review of the literature provides little or no satisfactory evidence of the essential nature of "bios" and only a series of widely conflicting statements regarding its chemical nature and properties.

Quite recently, Miss Copping [1929] has carried out in these laboratories a re-investigation of the fundamentally important question whether or not "bios" is necessary for the growth of yeasts in artificial sugar-salt media and has confirmed the observation that yeasts differ in this respect. Some species are able to reproduce indefinitely in artificial media, whilst others are unable to grow unless a factor resembling that originally described by Wildiers is supplied. *Saccharomyces cerevisiae*, the yeast most frequently employed in investigations on "bios," is definitely in the second class.

It next became of interest to re-examine the chemical nature of this substance, particularly in view of the claims that have been made regarding its isolation. The investigation reported in this paper concerns two main points, namely, the relation, if any, between "bios" and vitamin B, and the actual chemical nature of "bios" itself.

With regard to the first question, it will be recalled that Funk and Dubin [1921] were the first to suggest that the substance stimulating the growth of yeast also played an important part in the nutrition of animals. For a time active discussion took place over the possible identity of "bios" with vitamin B [see review of literature by Kruse and McCollum, 1929], with the result that they were finally regarded as distinct. The discussion has been revived, however, quite recently in the light of the discovery that the so-called vitamin B is a complex of at least two substances, of which one bears a superficial resemblance to "bios" in being stable to heat and alkali.

Regarding the nature and properties of "bios," there is, in the first place, the original description by Wildiers, who stated that it is an organic substance, soluble in water and 80 % alcohol, stable to heat and acid, not precipitated by neutral or basic lead acetate, phosphotungstic acid, phosphomolybdic acid, or by silver nitrate in acid solution or in the presence of ammonia. Most of these statements were supported by Devloo [1906], who added the view that the active substance is related to choline and possibly associated with choline in nature.

Both Wildiers [1901] and Funk and Dubin [1920] tested a series of purines and pyrimidines, but failed to find that any of them would replace "bios." The work of Fränkel and Schwarz [1920] and of Suzuki *et al.* [1923, 1925] tended to strengthen both the growing opinion that "bios" was a nitrogenous base, and the view that it is related to choline, although Fränkel and Scharf [1921] actually found that choline itself inhibits yeast growth.

On more than one occasion the study of the chemical nature of "bios" has been complicated by the suggestion that it is not a single substance [Fulmer, Duecker and Nelson, 1924]; a view which recently seemed to be established by the reported isolation of the constituents of "bios" by Miller [1924], of which one appears to have been identified as inositol [Eastcott, 1928].

One difficulty in accepting the idea of a complex "bios" was to reconcile it with the alleged activity of a single substance isolated by Eddy, Kerr and Williams [1924, 1926].

The "bios" of this group of investigators was described as a crystalline nitrogenous base, M.P. 223°, not showing the reactions of a primary amine, not precipitated by phosphotungstic acid [cf. Williams *et al.*, 1927], but yielding a crystalline benzenesulphonamide. On slender evidence it was believed to be related to proline. Particularly striking was their claim that whereas the benzenesulphonamide was inactive, the regenerated base showed an activity equal to that of the original "bios." Eddy and his collaborators also described the isolation of a second "bios" (β), apparently also a nitrogenous base, but this has not been obtained in a pure condition.

Furthermore, Kerr [1928] has recently announced the separation of another "bios" (also called β), a very hygroscopic nitrogenous base. It is claimed that this "bios" is also capable of acting by itself.

Further discussions of these claims would, therefore, appear to be more conveniently held over until the results of this investigation have been described.

EXPERIMENTAL.

Technique.

Throughout this investigation a strain of *Saccharomyces cerevisiae* (Chapman) No. 2190, National Collection of Type Cultures, was employed. It was maintained by regular subculturing on wort-agar slopes; cultures of 24–30 hours' growth being employed for "bios" experiments.

As an artificial medium for the "bios" tests, that described by Reader

[1927] was employed. Copping [1929] has shown that this medium is satisfactory for the purpose. "Bios" tests were always made by inoculating 25 cc. of sterile Reader's medium in 100 cc. Erlenmeyer flasks with a suspension of yeast in the medium, so that the original count[1] in the flask was of the order of 0·01, *i.e.* 2500 cells per cc. To certain of the flasks the sterile fractions to be tested were added, care being taken that the volume was not altered by more than 2 %. If there was likelihood of greater dilution, Reader's medium was employed to dissolve the substance to be tested.

All cultures were maintained at 22°. The growth was measured by cell counts made at intervals with the Thoma haemocytometer; all 400 small squares being counted in order to reduce the error. Growth was assumed to have occurred when there was multiplication of at least a hundredfold 24 hours after incubation, and when there was visible growth after 48 hours. The activity of the substance was measured as the least amount which produced this order of growth.

In that part of the investigation concerned with the relation of "bios" to vitamin B_2, fractions were examined for the latter factor. The technique of this test is described more fully elsewhere [Narayanan and Drummond, 1930]. Briefly, it consisted in observing whether the growth of young rats fed on a basal diet adequate in all respects other than a deficiency of vitamin B_2 was stimulated by administering the fraction.

Examination of known substances for "bios" activity.

As part of the investigation, it was thought to be worth while to examine a number of substances of known composition, that might, for one reason or another, be suspected of serving as "bios."

(*a*) *Nucleic acid and related substances.* In view of the work of Wildiers, and also of Funk and Dubin, referred to earlier, the action of yeast nucleic acid was investigated, but no stimulation of growth was observed following the addition of 0·01–0·1 mg. for each cc. of Reader's medium. Tests with the same concentration of guanine as hydrochloride, adenine as sulphate, and uracil also gave negative results; the very slight stimulation of yeast growth with the highest doses of guanine can probably be disregarded. These observations are in agreement with those of Wildiers.

(*b*) *Substances possibly related to vitamin B.* In view of the stability of "bios" to heat, and the recognition of the complexity of vitamin B, it was of interest to investigate whether the substances originally described by Funk [1912–13] as vitamin B, and the products, nicotinic acid and betaine obtained from it by Drummond and Funk [1914], represented any part of the vitamin B complex or possessed "bios" action. The former tests are described elsewhere, but as regards "bios" activity, no stimulation of yeast growth was produced by concentrations of 0·01–0·1 mg. per cc. of the original compound (M.P. 223°) of Funk, nicotinic acid, or betaine. The observations of Peters *et al.* [1928] that concentrates of vitamin B_1 may show power to stimulate the growth of yeast and micro-organisms has been confirmed, for it has been possible through the kindness of Dr Guha to test the materials obtained by him during the fractionation of the vitamin B_1 constituent of the vitamin B complex [Guha and Drummond, 1929]. Some of the earlier fractions tested showed a marked "bios" effect in concentrations of the order of 0·08 mg./cc., but, as the concentration of the vitamin progressed, the "bios" activity decreased. At the stage of the picrolonic acid fractionation no appreciable "bios" activity was shown by Dr Guha's concentrates.

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[1] Unit count = 250,000 yeast cells in 1 cc. of culture medium.

(*c*) *Substances related to choline.* In view of the original statement of Devloo, an examination was undertaken to ascertain whether choline, or any of the bases derived from other phosphatides, showed "bios" action under the conditions of our tests. Tests were made on purified preparations of lecithin and kephalin, for which the writer is indebted to Dr H. J. Channon, added to the cultures in the form of highly dispersed 0·1 % emulsions, but without any increased growth of the yeast being observed. The products of hydrolysis of these preparations of lecithin and kephalin were also found to be inactive in the same concentrations, whilst, at the suggestion of Dr O. Rosenheim, aminoethyl alcohol, for a pure specimen of which the writer is again indebted to Dr Channon, was tested in concentrations of 0·02–0·07 mg./cc., without any activity being observed. No activity was shown by phrenosin, sphingomyelin and the base sphingosine, tested in concentrations of 0·005–0·10 mg. In view of these results it appears probable that the activity of Devloo's material was due to an impurity and that he was incorrect in relating "bios" to choline and lecithin.

(*d*) *Miscellaneous substances.* The following substances were tested for reasons which need not now be given, since they were all found inactive in doses ranging from 0·005 to 0·1 mg./cc.: stachydrine, lysine, spermine, guanidine, methylguanidine, putrescine, cadaverine, hexosediphosphate (fermentation) and potassium pyrophosphate. Cysteine in a concentration of 0·002–0·01 mg./cc. and the three dyes, methylene blue, potassium indigotetrasulphonate and indigo carmine in doses of 0·001–0·005 mg./cc. were also found inactive.

(*e*) *Tests on Eddy's preparations.* Through the kindness of Prof. Eddy the writer was provided with small quantities of both the α- and the β-"bios" isolated in his laboratories. They were tested in concentrations ranging from 0·025 to 0·075 mg./cc. The highest doses of both preparations produced a slight stimulation of growth of yeast, but the smaller concentrations were, according to the standards used in this investigation, inactive (see Table I). Thus, for example, after 120 hours' incubation the count in the flasks to which 0·075 mg./cc. of α-"bios" had been added was only 20, whilst that in the case of the β-"bios" was 24. These figures compare unfavourably with many of our impure concentrates, which in the same interval of time produce a count of

124 with a concentration of not more than 0·02 mg./cc. Judged by the standard adopted in this investigation the preparations of Eddy would be regarded as substances of relatively poor "bios" activity.

Table I.

Source of "bios"	Dose per cc. of medium (mg.)	Initial inoculation count/cc.	Growth in units of count after			
			24 hr.	48 hr.	72 hr.	120 hr.
Negative control	—	0·01	0·01	0·02	0·02	0·04
,, ,,	—	0·10	0·10	0·40	0·90	2·40
Eddy's α-"bios"	0·025	0·01	—	—	0·50	6·80
	0·025	0·10	0·10	0·50	2·80	17·00
	0·050	0·01	0·02	0·07	1·80	14·00
	0·050	0·10	0·20	0·50	4·30	19·50
	0·075	0·01	0·03	0·06	2·00	20·00
	0·075	0·10	0·40	0·90	3·90	26·00
Eddy's β-"bios"	0·025	0·01	0·01	—	0·40	7·30
	0·025	0·10	0·10	0·30	3·20	19·00
	0·050	0·01	—	—	—	16·80
	0·050	0·10	0·20	—	—	24·00
	0·075	0·01	0·01	0·05	1·90	24·00
	0·075	0·10	0·30	0·70	3·50	28·40
Extract (*d*) Table II	0·050	0·01	2·30	—	24·00	209·00
	0·050	0·10	5·20	14·70	—	—
Extract (*e*) Table II	0·020	0·01	1·90	—	21·00	172·00
Extract (*h*) Table II	0·010	0·01	1·00	—	9·60	102·50
	0·020	0·01	1·40	—	13·70	124·00

As it was possible that these differences might be due to the fact that Eddy and his co-workers used a higher incubation temperature (31°), a few experiments were made to control this point. The following results show that the different temperature does not explain the discrepancy.

Temperature of incubation 31°.

Source of "bios"	Dose per cc. of medium (mg.)	Initial inoculation count/cc.	Growth in units of count after			
			24 hr.	48 hr.	72 hr.	120 hr.
Negative control	—	0·01	0·01	—	0·02	0·04
Eddy's α-"bios"	0·075	0·01	0·02	0·07	3·20	28·80
	0·075	0·10	0·20	0·60	5·30	34·60
Eddy's β-"bios"	0·075	0·01	0·02	0·09	4·10	27·60
	0·075	0·10	0·30	0·90	4·80	23·30
Extract (*d*) Table II	0·050	0·01	3·20	8·90	—	222·50

Fractionation of yeast extracts for "bios."

(*a*) *Preparation of yeast extract.* A series of experiments was made to ascertain the most suitable means of extracting "bios" from brewer's yeast. Extraction with alcohol of various strengths, the use of acid and alkaline alcohol and the effect of different temperatures were all investigated, but no outstanding advantage in any one method was discovered. The method finally adopted was as follows.

Brewer's yeast was extracted in 7 or 14 lb. batches with 2·5 or 5 litres of alcohol for 4 hours at 60 to 70°. After filtration, the pressed residue was twice re-extracted with 50 % alcohol. The united extracts were concentrated under reduced pressure.

In some cases it was found convenient to use as raw material a commercial yeast extract (marmite), although an obvious drawback to this procedure was the presence in the extract of substances other than those originally present in the yeast.

The original yeast extracts usually produced a count of 16–20 in 48–60 hours when added in concentrations of 0·3–0·4 mg./cc.

(*b*) *Alkaline hydrolysis.* The concentrates from yeast or yeast extract (marmite) were hydrolysed by heating for 2 or 3 hours in an autoclave at 15 lb. pressure with a 20 % solution of barium hydroxide. The precipitate formed during the hydrolysis was removed by filtration, and the filtrate rendered free from barium by addition of a slight excess of dilute sulphuric acid. The barium sulphate precipitate carried down a considerable quantity of organic material from the solution at this reaction (p_H 6·8), but the filtrate contained practically all the "bios."

The dose of this preparation required to produce yeast growth of 16–20 counts in 48–60 hours was usually 0·2–0·35 mg./cc. of medium.

(*c*) *Precipitation with neutral lead acetate.* The filtrate from the barium sulphate precipitate was treated with an excess of lead acetate solution, about 110 g. being usually employed to treat the material obtained from 7 lb. of yeast, and the mixture allowed to stand overnight. After filtration both filtrate and precipitate were treated with H_2S to remove lead, the lead-free liquids brought to a reaction of p_H 6·8 and concentrated.

The "bios" activity was found exclusively in the lead acetate filtrate, of which the dose was found to be 0·15–0·25 mg./cc., whereas in confirmation of Rosedale [1927] and of Chick and Roscoe [1929] it was found that the vitamin B_2 was separated almost completely in the lead acetate precipitate.

From this point of the investigation, having established that "bios" and vitamin B_2 are not identical, the two substances were studied separately.

(*d*) *Attempts to adsorb "bios" on norite charcoal.* Following the steps that have been useful in effecting a concentration of certain of the B vitamins, attempts were made to adsorb "bios" by treating the filtrate from the treatment with lead acetate (*c*) with adsorbent charcoal (norite) at different reactions. The reaction of the liquid was adjusted by units of p_H in stages from 2 to 7, at each stage being shaken with appropriate quantities of norite. The charcoal was filtered off and extracted with warm 50 % alcohol containing 1 % HCl, but no evidence of an adsorption of "bios" was obtained. After repeated trials this step was omitted from the later processes.

(*e*) *Treatment with* 80 % *alcohol.* The final filtrate from the "norite" treatment, or, in the later processes, the filtrate from the lead acetate precipitation was evaporated under reduced pressure to a thick golden brown syrup, and extracted thrice with 5 volumes of hot 80 % alcohol. This treatment removed a quantity of insoluble inorganic substances and other inactive materials, and effected a certain amount of concentration of "bios"; the dose being reduced to 0·07–0·10 mg./cc.

(*f*) *Precipitation with phosphotungstic acid.* The 80 % alcohol extract was concentrated under reduced pressure to remove the alcohol, the thick brown concentrate dissolved in 500 cc. of 5 % sulphuric acid, and treated with an excess of a saturated solution of phosphotungstic acid in 5 % sulphuric acid; a 25 % excess of the precipitant is necessary if the precipitation of "bios" is to be satisfactory. The mixture was allowed to stand overnight in the dark and filtered. Both filtrate and precipitate were treated with barium hydroxide to remove phosphotungstic and sulphuric acids. It was found that under these conditions "bios" is almost completely precipitated by phosphotungstic acid; the activity of the material present in the decomposed precipitate being 0·008–0·02 mg./cc. The filtrate showed insignificant activity.

(*g*) *Silver fractionation.* Two methods of fractionating by means of silver the materials precipitated by phosphotungstic acid were employed.

In the first place a series of fractions with silver nitrate were obtained at different reactions, baryta being employed to lower the acidity by stages. Precipitates were removed at p_H 2 (Ag I), p_H 4·6 (Ag II), p_H 6·8 (Ag III), p_H 8 (Ag IV) and finally after saturation with baryta (Ag V). Each precipitate was suspended in hot water and decomposed with hydrochloric acid gas, but no appreciable "bios" activity was traced in any one; the final filtrate from the silver fractions, rendered free from silver and barium by sulphuric acid, showed considerable potency, being active in doses of 0·01–0·02 mg./cc.

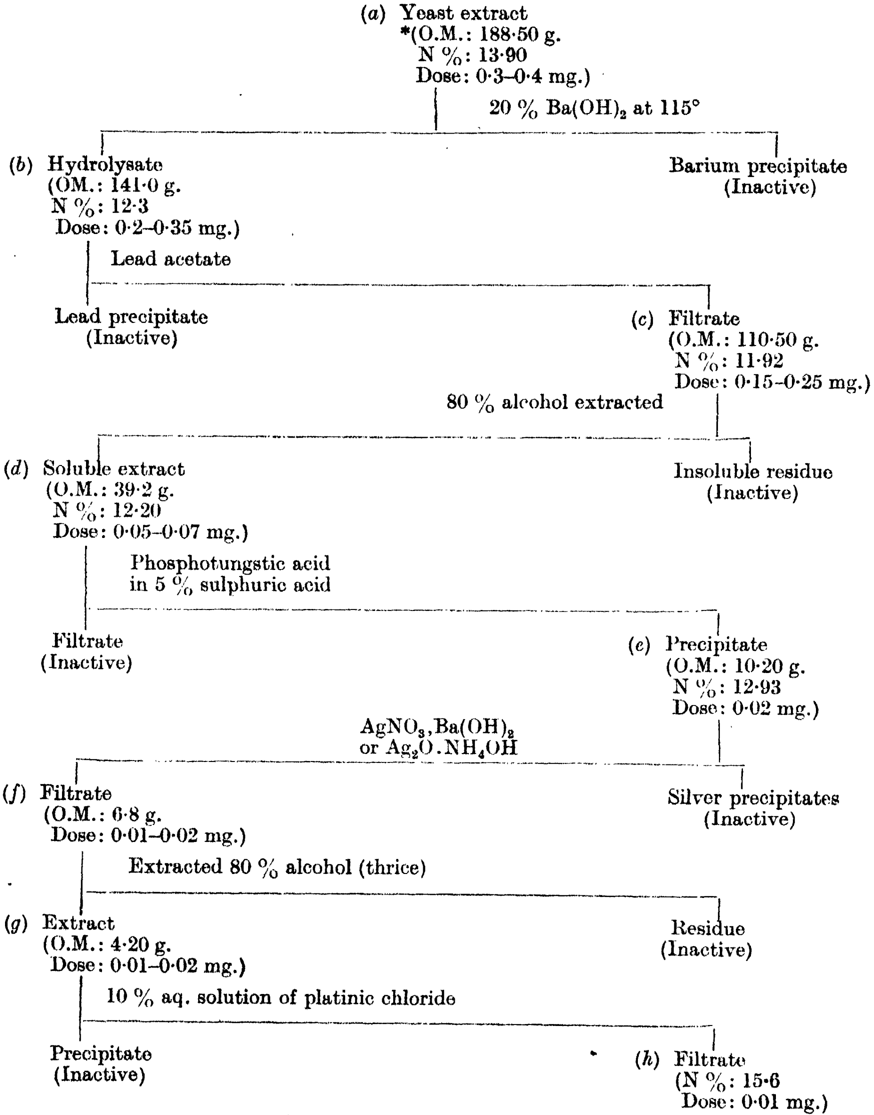
The alternative treatment with silver consisted of rendering the diluted solution from *e* (Table II) distinctly alkaline by means of ammonia and adding freshly precipitated silver oxide until maximal precipitation occurred. The "bios" activity was not removed in the heavy precipitate, but remained in the filtrate after removal of ammonia and the excess of silver. The activity of this preparation was of the same order, namely, 0·01–0·02 mg./cc.

(*h*) *Treatment with platinic chloride.* The filtrates from the silver treatments on concentration to dryness yielded semi-solid materials from which a further amount of inactive inorganic substances was removed by extraction with 80 % alcohol. The solution after removal of alcohol on the addition of a 10 % aqueous solution of platinic chloride yielded a crop of a brown crystalline solid. The crystals were removed, and well washed with water, in which they were relatively insoluble, and decomposed with H_2S. The material precipitated by platinum showed "bios" activity, but only in relatively high concentrations (0·10–0·20 mg./cc.). Most of the activity was found in the platinum filtrate of which the active concentration was about 0·01 mg./cc.

A number of attempts have been made to concentrate further the "bios" present in the final filtrate from the platinum treatment, but with no appreciable lowering of the concentration necessary to produce the required stimulation of yeast growth.

The progress of a typical fractionation is illustrated in Table II.

Table II.



* O.M. = organic matter. Dose = active dose for 1 cc. of medium.

Properties of the active concentrate.

The material obtained by the decomposition of the platinum filtrate contains 15·6 % of nitrogen (calculated on an ash-free basis) but no phosphorus. It does not give the following tests for various types of nitrogenous compounds: carbylamine reaction, nitrosamine reaction, biuret test, Folin-Denis test, Millon reaction, ninhydrin reaction and Denigè's test, but it gives a fairly strong reaction with Pauly's reagent. No precipitates were obtained on the addition of aqueous or alcoholic picric acid, mercuric sulphate (Hopkins's reagent) or mercuric chloride. Bromine is absorbed with great avidity, yielding a gummy resinous product.

The "bios" activity is inappreciably affected by treatment with nitrous acid or dilute nitric acid, but oxidation by warming with hydrogen peroxide for 30 minutes causes inactivation. All efforts to prepare a solid benzenesulphonamide failed, and no further evidence has yet been obtained to throw light on the chemical nature of the active substance.

Comparison of the various "bios" preparations.

The more important statements regarding "bios" are summarised in Table III; it being remembered that according to Miller [1924] the growth of yeast in a sugar-salt medium is not satisfactory unless "bios I" is added to "bios II." The former has been identified by Eastcott [1928] as inositol; furthermore, the "bios II" of these workers is described as extremely soluble in acetone, readily removed from solution by shaking with charcoal, and not precipitated by barium hydroxide [Lucas, 1924; Miller, 1924].

Table III.

	Wildiers, Devloo	Eddy and his co-workers	Author's concentrate
Solubilities:			
Water	Soluble	Soluble	Soluble
Alcohol (abs.)	—	Sol. in 95 %	Sparingly sol.
,, (80 %)	Soluble	Soluble	Soluble
Acetone	—	Soluble in aqueous acetone	Insoluble
Ether and chloroform ...	Insoluble	—	Insoluble
Stability to reagents:			
Alkalis (hot or cold baryta and NaOH)	Stable	Stable	Stable
Acids (cold HCl, H_2SO_4, HNO_3)	Stable	Stable	Stable
Hot HCl, HNO_3 and H_2SO_4	Stable	—	Stable
Nitrous acid	—	—	No action
Hydrogen peroxide ...	—	—	Inactivated
Precipitants:			
Lead acetate (neutral) ...	Not precipitated	—	Not precipitated
,, (basic) ...	Not precipitated	—	Not precipitated
Silver nitrate (acid) ...	Not precipitated	—	Not precipitated
,, (alkali)	Not precipitated	—	Not precipitated
Phosphotungstic acid ...	Not precipitated	Not precipitated	Precipitated
Mercuric sulphate ...	Not precipitated	—	Not precipitated
Mercuric chloride ...	Not precipitated	—	Not precipitated (?)
Platinic chloride (aqueous)	Not precipitated	—	Not precipitated
,, (alcoholic)	Not precipitated	—	Not precipitated
Picric acid	Not precipitated	—	Not precipitated
Nature	Believed to be an org. N base	Nitrogenous base	Simple N compound

It will be seen that there is some disagreement regarding the properties of "bios." Actually, the disagreement is more serious than is indicated by the comparison of the properties of the various preparations, because Lucas [1924] records a failure to observe under the conditions of his test any stimulant action with Eddy's "bios," and with our technique the preparations of Eddy were also found to be virtually inactive (see Table I).

The identification of "bios I" as inositol by Miss Eastcott [1928] has also complicated the question considerably. During the course of the investigation being reported a large number of experiments has been made to ascertain whether the activity of any of the fractions was dependent upon or was augmented by the addition of inositol. In no single case was this found to be the case; the following examples are typical (Table IV).

Table IV.

(Initial inoculation: 0·01 count/cc., incubation temperature: 22°.)

Inositol (Basle) mg./cc.	Inositol (Pryde) mg./cc.	"Bios" extract mg./cc. Table II	Growth in units of count after			
			24 hr.	48 hr.	72 hr.	120 hr.
Nil	Nil	Nil	0·01	0·01	0·02	0·04
0·02	,,	,,	0·01	0·01	0·02	0·04
0·04	,,	,,	0·01	0·02	0·02	0·04
0·08	,,	,,	0·01	0·02	0·04	0·04
0·10	,,	,,	0·01	0·02	0·04	—
0·20	,,	,,	0·02	0·04	0·07	0·18
Nil	0·05	,,	0·02	0·05	0·15	0·20
,,	0·10	,,	0·02	0·04	0·10	0·20
,,	0·20	,,	0·03	0·09	0·15	0·30
0·02	0·20	0·050 (*d*)	2·30	5·40	21·80	—
0·08	0·20	0·050 ,,	2·10	6·20	—	210·00
0·10	0·20	0·050 ,,	1·90	5·70	—	206·50
0·20	0·20	0·050 ,,	2·10	5·90	23·00	—
Nil	0·20	0·050 ,,	2·40	6·10	20·20	205·00
,,	0·050	0·020 (*e*)	1·80	5·00	20·50	—
,,	0·10	0·020 ,,	1·90	5·30	—	184·00
,,	0·20	0·020 ,,	—	4·70	18·30	—
,,	Nil	0·020 ,,	1·75	4·80	19·70	—
,,	0·05	0·010 ,,	0·70	2·00	—	78·00
,,	0·10	0·010 ,,	0·85	—	—	82·00
,,	0 20	0·010 ,,	0·80	2·70	—	85·50
,,	Nil	0·010 ,,	0·70	2·20	—	80·40

(Initial inoculation: 0·10 count/cc.)

Inositol (Basle) mg./cc.	Inositol (Pryde) mg./cc.	"Bios" extract mg./cc. Table II	24 hr.	48 hr.	72 hr.	120 hr.
Nil	Nil	Nil	0·10	0·20	—	0·40
0·02	,,	,,	0·10	0·20	—	0·50
0·05	,,	,,	—	0·20	—	—
0·10	,,	,,	—	0·20	—	—
0·20	,,	,,	0·20	—	—	1·50
0·02	,,	0·020 (*e*)	4·50	19·50	—	—
0·05	,,	0·020 ,,	4·90	22·30	—	—
0·10	,,	0·020 ,,	4·70	20·70	—	—
0·20	,,	0·020 ,,	4·60	20·80	—	192·00
Nil	0·10	Nil	0·40	1·20	—	—
,,	0·20	,,	0·50	1·40	—	3·00
,,	0·05	0·020 (*e*)	4·80	—	—	187·00
,,	0·10	0·020 ,,	—	—	67·90	—
,,	0·20	0·020 ,,	5·70	22·60	—	—
,,	Nil	0·020 ,,	4·70	21·50	—	186·00

A series of experiments was also conducted at 28°, the temperature of incubation employed by Miss Eastcott [1928], but the results were not essentially different from those given above. Of the same character were the results of a series of experiments with varying doses of "bios" extracts (Table V).

Table V.

(Initial inoculation: 1·0 count/cc., incubation temperature: 22°.)

Inositol (Basle) mg./cc.	Inositol (Pryde) mg./cc.	"Bios" extract mg./cc. Table II	Growth in units of count after	
			24 hr.	48 hr.
Nil	Nil	Nil	1·0	2·80
0·05	,,	,,	—	3·20
0·10	,,	,,	—	4·60
0·20	,,	,,	2·30	5·20
0·05	,,	0·020 (*h*)	8·70	32·00
0·10	,,	0·020 ,,	8·60	31·70
0·20	,,	0·020 ,,	9·20	33·90
Nil	0·05	0·020 ,,	7·80	29·20
,,	0·10	0·020 ,,	8·70	33·50
,,	0·20	0·020 ,,	8·50	31·00
,,	0·05	Nil	—	2·90
,,	0·10	,,	—	5·70
,,	0·20	,,	4·00	9·20 (?)
,,	Nil	0·020 (*h*)	7·90	31·00
,,	,,	0·010 ,,	4·80	19·40

One sample of inositol employed was a preparation made by the Society of Chemical Industry of Basle. It was purified by several recrystallisations from dilute alcohol and melted sharply at 215°. A few experiments were also made with a preparation isolated from mammalian muscle, for which I am indebted to Dr J. Pryde. All the experiments with inositol proved negative in the sense that no effect on the growth of yeast was observed.

The active concentrates obtained in this investigation were examined qualitatively for inositol, but no indication of its presence was obtained. The conclusion reached is, therefore, that, if the methods of studying the growth of yeast employed by the Toronto investigators differ in no fundamental manner from those used in this investigation, the activity of the inositol observed by them must have been due either to the presence of an impurity, or to some such effect as has been recently described by Miss Reader [1929] in the case of mannitol. The present study provides no explanation of the different behaviour of inositol in these experiments and those of the Toronto investigators. Until a satisfactory explanation of the discrepancies is forthcoming it might be assumed that different yeasts require different types of "bios," although the writer's experience is against making such an assumption. Miss Copping [1929] was good enough to test a number of the active concentrates and found that they stimulated the growth of a wide variety of yeasts of different types.

DISCUSSION.

Until the investigation has been carried further, it is undesirable to attempt to reconcile the many conflicting statements regarding "bios" that are to be found in the literature.

For the present it will be sufficient to state that concentrates have been prepared from yeast which are highly active in stimulating the growth of *S. cerevisiae* in an artificial sugar-salt medium. These concentrates exhibit an activity in doses that are considerably smaller than those recorded by Eddy for his preparations, one of which is believed to be a pure substance, or those described by the group of Toronto workers. In this investigation no evidence has been obtained to support the activity of Eddy's preparations of α- and β-"bios," nor are the results described in this paper in agreement with the statements regarding the activity of the "bios I" and "bios II" of Miller, Lucas and others.

The properties of the most active concentrates prepared during the course of this work suggest that the active material is a comparatively simple nitrogenous substance[1].

SUMMARY.

(1) "Bios" and vitamin B_2 are not identical. A chemical separation of vitamin B_2 from "bios" has been effected.

(2) A series of substances of known chemical composition has been tested for "bios" activity. None of these substances has been found to exert a marked stimulating influence on the growth of the yeast.

(3) A method of fractionation of "bios" is described, which provides concentrates producing marked stimulation of yeast growth in doses of the order of 0·01 mg./cc. of an artificial sugar-salt medium. The final concentrate obtained appeared to consist largely of relatively simple nitrogenous substances and contained no phosphorus.

(4) The properties and activity of this concentrate are compared with those of Eddy's preparations, and also with those of the preparations of Miller, Lucas and their colleagues. The conclusion is reached that a more active preparation has been obtained than those described by them, and, therefore, that their claims to have isolated "bios" cannot at this stage be admitted.

(5) An extensive examination of Eastcott's statement that inositol is an essential unit of "bios" has been made, but no evidence was obtained to support her claim.

In conclusion, the writer wishes to make grateful acknowledgment to Prof. J. C. Drummond for his kind interest and advice throughout the course of this investigation.

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[1] Since this typescript was prepared for publication a paper by Williams, Warner and Roehm [*J. Amer. Chem. Soc.* 1929, **51**, 2764] has appeared where the authors record results very similar to those obtained in the foregoing paper on Eddy's "bios" preparations and inositol. Williams and his co-workers claim to have obtained a very concentrated form of "bios" (their "Z" concentrate adsorbable by fuller's earth) which, however, manifests its greatest activity only in the presence of another factor present in the residue after adsorption by fuller's earth. The "Z" concentrate is claimed to produce definite stimulation of growth in as low a concentration as 0·00005 mg./cc. when duly supplemented by 0·8 mg./cc. of the unadsorbed residue. As has already been shown in the foregoing paper no evidence in support of the complex nature of "bios" has been obtained during the course of the present investigation.

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III. THE CONCENTRATION OF VITAMIN B_2.

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While a considerable amount of work has been done to elucidate the chemical nature of vitamin B_1 as yet few observations of a similar character have been recorded regarding the more recently discovered vitamin B_2. Of the few facts known concerning the latter substance, the most important are those that indicate its comparative stability to heat and to somewhat drastic treatment with aqueous alkalis. Several investigators have stated that it is sparingly soluble in alcohol of greater concentration than 80 %, while it has also been recorded that it is absorbed by fuller's earth [Salmon *et al.*, 1928] and silica gel [Levene, 1928]. The attempts to separate the two vitamins B_1 and B_2 have been based on these properties, but so far little progress has been made in the isolation of vitamin B_2. The most potent concentrate recorded in the literature appears to be that obtained by Levene [1928], by means of adsorption on silica gel. This preparation supplied the requirements of the young rat in doses of only 2 mg. of organic matter daily. He observed that the activity was destroyed by treatment with nitrous acid.

The present investigation was undertaken as part of the general study of vitamin-like substances present in yeast extracts.

Experimental.

In the foregoing paper a description is given of an investigation on the chemical nature of "bios." During the earlier part of this investigation it was uncertain whether the yeast stimulant was identical with the factor known as B_2 or not, but it was soon ascertained that precipitation of the baryta-hydrolysates of yeast extracts with lead acetate effected a separation of the two substances. The vitamin B_2 is precipitated by lead acetate whilst "bios" remains in the filtrate. The precipitation of vitamin B_2 by this reagent has been recorded by Rosedale [1927], and by Chick and Roscoe [1929]. The present communication describes the efforts that were made to separate the factor B_2 from the materials precipitated by lead acetate (see Table II [Narayanan, 1930]).

A. *Technique.* In these experiments young rats (usually black and white, bred in these laboratories) of approximately 50 g. in weight were placed on a vitamin-free diet consisting of caseinogen 20 %, starch 74 %, and a special salt mixture 4 %. They were also given 40 mg. daily of cod-liver oil of tested vitamin A and D strength and each rat was also given a supply of a concentrate containing vitamin B_1 sufficient to permit satisfactory growth if the diet was otherwise adequate. The concentrate was made by the method of Kinnersley and Peters [1927].

Each rat was kept in a separate cage and when the growth had quite, or very nearly, ceased, a daily dose of the extract to be tested was given. An extract under test was assumed to be active when the rats grew at the rate of at least 10 g. a week.

B. *Tests on known substances.* A number of substances, which for one reason or other might possibly have been suspected of being related to vitamin B_2, were tested. All these tests proved negative. The list of substances tested is given below with the doses employed.

Table I.

Substance	Dosage (mg.)	Average growth in a week (g.)
Nucleic acid (yeast)	1–10	– 2
Guanine hydrochloride	1–5	Nil
Adenine sulphate	1–5	,,
Funk's compound, M.P. 234°	1–5	1
Nicotinic acid hydrochloride	1–5	Nil
Betaine hydrochloride	1–5	– 1
Inositol	1–10	– 1 to + 1
Potassium pyrophosphate	1–10	– 3
Non-saponifiable matter from yeast fat	1–5	Nil

C. *Preparation of lead acetate fraction.* The precipitate produced by the addition of lead acetate to the extract hydrolysed by alkali, or to the aqueous solution of the material extracted from yeast by alcohol [see Narayanan, 1930] was decomposed by suspending in warm water and slowly adding 10 % sulphuric acid while the mixture was stirred mechanically. When acid to Congo red, the lead sulphate was removed by filtration, and the filtrate neutralised with sodium hydroxide. The filtrate fraction was found to be active and promoted growth in daily doses representing 10–15 mg. of organic matter. (N content 6·7 %.) (Curve no. I, Fig. 1.)

The filtrate containing material not precipitated by lead acetate produced practically no growth, even in daily doses as high as 400 mg. of organic matter.

D. *Adsorption on fuller's earth.* Preliminary experiments were made to discover if the factor is readily adsorbed by fuller's earth, and, if so, the best conditions for effecting concentration by this means. A preliminary series of trials with the liquid resulting from decomposition of the lead acetate precipitate demonstrated that over a range of p_H extending from 6·8 to 0·1 the adsorption increased as the acidity was raised. At p_H 0·1 the removal of the active factor by fuller's earth was almost complete, and the main bulk of a large preparation was consequently treated in the following manner.

The active liquid from the lead acetate precipitate was rendered approximately 0·9 *N* with sulphuric acid and treated with 3 g. of fuller's earth for every 100 cc. of the liquid.

The mixture was well stirred mechanically for half an hour, filtered and the earth well washed with a small quantity of 0·9 *N* sulphuric acid. The filtrate and washings were similarly treated a second time with 1·5 g. of fuller's earth for each 100 cc. Approximately 40 % of the total solids and organic matter of the original extract was adsorbed. The resulting earth contained practically all the active factor, and promoted growth in doses of 40 mg. of earth; corresponding to 6 mg. of adsorbed organic matter. (Curve no. II, Fig. 1.)

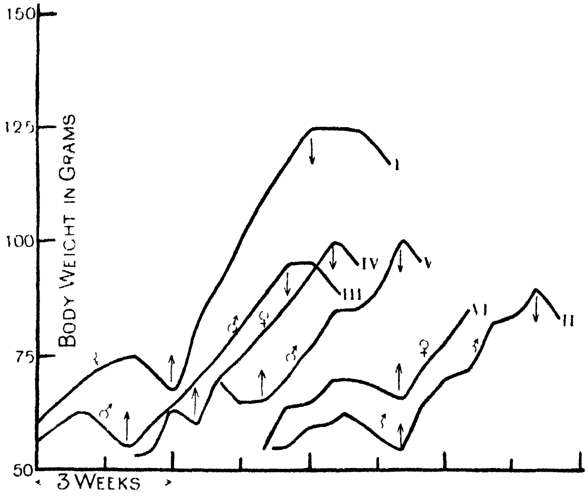


Fig. 1.

↑ = Supplement given. ↓ = Supplement withdrawn.

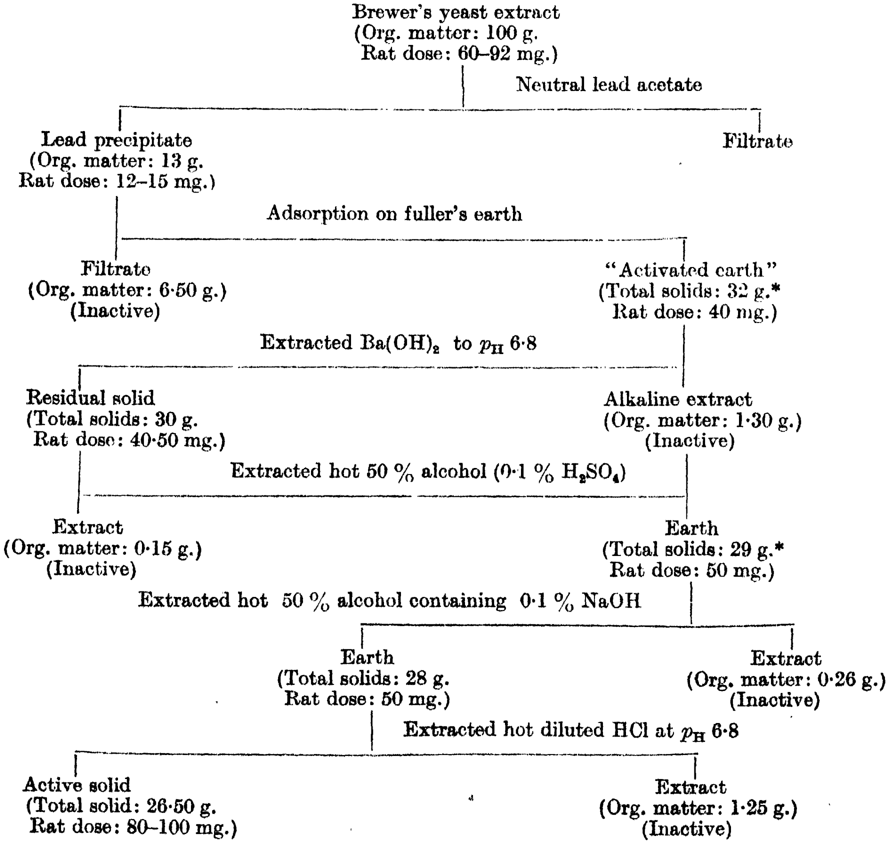
The "activated" fuller's earth was now triturated with cold saturated baryta until alkaline to bromocresol purple, filtered, and the precipitate well washed with distilled water until the washings failed to give a test for barium. The filtrate and washings contained 26 % of the solids adsorbed on the earth, but did not promote growth when tested. On the other hand, the earth after extraction with baryta produced growth in doses of 40 to 50 mg. of earth, corresponding to 5 mg. of residual adsorbed organic matter. (Curve no. III, Fig. 1.)

The earth was now extracted with 50 % alcohol containing 0·1 % of sulphuric acid. Twenty-six g. of earth, containing approximately 2·7 g. of organic matter, were twice extracted at 60 to 70° for 1 hour with 50 cc. of the acid alcohol. The extracts and washings were evaporated at reduced pressure, and the residual fluid carefully neutralised. On testing the extract it was found to be inactive. It contained 0·16 g. of organic matter, representing 6 % of the total adsorbed substances. The residual fuller's earth was found to be still active in doses of 50 mg. of earth. (Curve no. IV, Fig. 1.)

The residual earth was now extracted in a similar manner with 50 % alcohol containing 0·1 % of sodium hydroxide. The extract after removal of the alcohol and neutralisation was found to contain 0·2 g. of organic matter, but to be quite inactive. The residual solid weighed 20·5 g. and still retained its original activity, *i.e.* 50 mg. of earth. (Curve no. V, Fig. 1.)

A quantity of this residual "activated" earth, representing a total of approximately 400 daily doses, was again extracted, this time with dilute HCl at a p_H of 6·8, under the same conditions as before. The extract was concentrated and neutralised. It contained 0·75 g. of organic matter, but was inactive. The activity of the earth was found to have been somewhat reduced by this treatment as it required doses of 80–100 mg. to produce an average growth of 10–12 g. a week. (Curve no. VI, Fig. 1.)

The following scheme illustrates the experiments described.



* Total solids = total weight of "activated" fuller's earth.

E. *Adsorption on "norite" charcoal.* In view of the failure that attended the efforts to concentrate vitamin B_2 by the use of fuller's earth, an attempt was made to adsorb the active factor on "norite" charcoal. After a series of trials the following procedure was adopted.

The extract from the decomposition of the lead acetate precipitate from 7 lb. of yeast was rendered approximately 0·9 N with sulphuric acid, and treated with 5 g. of norite for every 100 cc. of the liquid. The mixture was well stirred for half an hour and filtered. The charcoal was then washed well with 0·9 N H_2SO_4, and the filtrate and the washings were again treated with 2 g. of charcoal per 100 cc. The combined charcoals weighed 42 g. when dry. The filtrate and washings were reserved for further experiment.

The charcoal fraction was suspended in 50 cc. of a solution of 1 % sodium hydroxide in 50 % alcohol, added until the reaction of the mixture was p_H 6·8, and the mixture heated on the water-bath for 1 hour, care being taken to keep the reaction at p_H 6·8. The alcoholic solution was filtered and concentrated under reduced pressure. The concentrate contained 40 % of the organic matter of the original extract, but was practically inactive. The residual charcoal, however, showed slight activity in 60 mg. doses. (Curve no. VII, Fig. 2.)

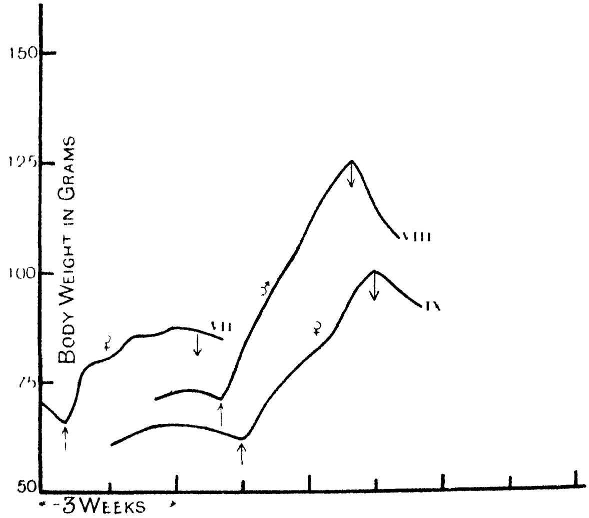


Fig. 2.

↑ = Supplement given. ↓ = Supplement withdrawn.

The reaction of the filtrate and washings from this charcoal treatment was adjusted to p_H 2·3 by the addition of sodium hydroxide and a similar treatment with norite carried through. The combined charcoals were similarly extracted at 60–70° with 50 % alcohol at p_H 6·8. The alcoholic extract contained 50 % of the residual organic matter, but did not produce any appreciable growth in 5–6 mg. doses. The extracted charcoal was quite inactive. It was concluded that norite does not appreciably adsorb vitamin B_2 at this p_H.

The filtrate from the charcoal treatments was concentrated under reduced pressure to a thick syrup and extracted with hot 50 % alcohol. A granular precipitate consisting mostly of inorganic matter separated, and was discarded on being found to be quite inactive. The alcoholic extract contained practically all the residual organic matter but was also inactive. The loss of activity in the experiment has not been satisfactorily accounted for.

F. *Attempts to concentrate vitamin B_2 by means of alcohol.* In view of the failure of the efforts to concentrate vitamin B_2 by adsorption methods, it was decided to attempt fractionation by alcohol. The fraction resulting from decomposition of the lead acetate precipitate was treated with absolute alcohol until the solution contained approximately 50 %. A considerable amount of a slimy precipitate was obtained which was filtered off and washed with small quantities of 50 % alcohol. The alcoholic extracts and washings were concentrated under reduced pressure. This extract was found to be active in daily doses of 9 mg. of organic matter (curve no. VIII, Fig. 2), and contained about 88 % of the activity of the original fraction.

The 50 % alcoholic extract was further treated by raising the percentage of alcohol to approximately 70 %, when another slimy dark-brown precipitate was deposited. This was removed by filtration and well washed with small quantities of 70 % alcohol. The filtrate was concentrated, but on being tested on rats was found to be inactive. The precipitate contained most of the activity, giving good growth in daily doses of 6 mg. of organic matter [cf. Sherman and Sandels, 1929]. Further investigation of this fraction is in progress. (Curve no. IX, Fig. 2.)

Some properties of vitamin B_2.

(*a*) *Treatment with acids and alkalis.* In the course of these experiments, further confirmation was obtained of the observation that the active factor, vitamin B_2, withstands rather drastic treatment with acids and alkalis.

No appreciable destruction appears to occur when either a yeast extract or brewer's yeast is hydrolysed by 10–15 % sulphuric or hydrochloric acid at boiling point for 24 hours, or when they are treated with 10–15 % barium hydroxide solution in an autoclave at 110–120° for 1 to 3 hours. (Curves nos. X, XI, XII, Fig. 3.)

(*b*) *Treatment with hydrogen peroxide.* The active factor is apparently stable to hydrogen peroxide. 100 cc. of the lead precipitate fraction, after removal of the lead, was treated with an excess of hydrogen peroxide, the mixture being warmed on the water-bath at 60–70° for 30 minutes. The resulting liquid was equal in activity to the original untreated material. (Curve no. XIII, Fig. 4.)

(*c*) *Treatment with nitrous acid.* In contradiction of Levene's statement [1928] and in confirmation of the more recent observation of Chick [1929] no appreciable destruction of the active factor was observed when treated with nitrous acid. 100 cc. of the lead precipitate fraction (containing 1·5 g. of organic matter) were treated with 2·5 g. of sodium nitrite and the mixture was cooled on ice. 10 % hydrochloric acid was then slowly added and the mixture allowed to stand for 10 minutes. The solution was then warmed to 60–70°, and finally neutralised with sodium hydroxide solution. The resulting liquid showed no appreciable deterioration of its original activity. (Curve no. XIV, Fig. 4.)

(*d*) *Solubility*. Apart from its extreme solubility in water, vitamin B_2 is very soluble in 50–60 % alcohol, but only sparingly so in alcohol of greater concentration, and practically insoluble in higher alcohols, of the type of butyl alcohol, as the following experiment shows.

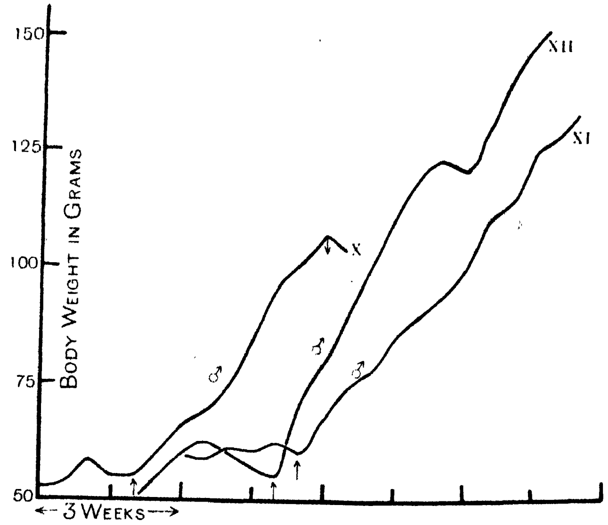


Fig. 3.

↑ = Supplement given. ↓ = Supplement withdrawn.

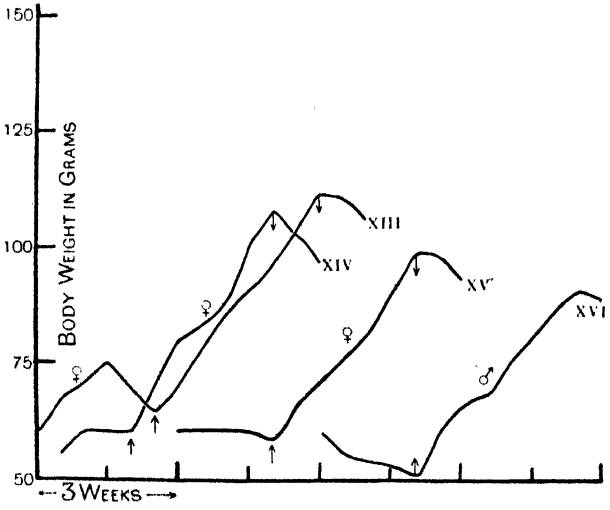


Fig. 4.

↑ = Supplement given. ↓ = Supplement withdrawn.

The extraction was made in a continuous extraction apparatus essentially of the form described by Dudley [1919].

The extract from 7 lb. of yeast was concentrated to a thick brown syrup, and extracted in two lots with *n*-butyl alcohol at 60–70° for 48 hours. The butyl alcohol extract was separated from the aqueous mixture, and the latter solution was again subjected to extraction for a further period of 48 hours. The extracts were combined and concentrated under reduced pressure to remove all the solvent. The concentrate contained 5 % of the total solids of the original extract, but was found useless as a source of vitamin B_2. The residual aqueous liquor retained practically all the activity. (Curves nos. XV and XVI, Fig. 4.)

SUMMARY.

(1) An attempt to concentrate vitamin B_2 by means of adsorption on fuller's earth is described. While adsorption was complete at the extreme acid range of p_H (0·05–0·10), great difficulty was encountered in removing the adsorbed active factor.

(2) "Norite" charcoal is not an efficient adsorbent of the active factor.

(3) The active factor is relatively insoluble in alcohol of greater concentration than 70 % by volume. By this process a concentrate has been obtained, of which the daily dose which supplies the requirements of the young rat contains 6 mg. of organic matter. Further fractionation of this concentrate is in progress.

(4) The general finding that the active factor is stable to heat, acids and alkalis is confirmed. It is also found that vitamin B_2 is not destroyed by either hydrogen peroxide or nitrous acid. It is not appreciably soluble in butyl alcohol.

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IV. THE RELATIVE VITAMIN A VALUE OF THE BODY AND LIVER OILS OF CERTAIN FISH.

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INTRODUCTION.

In view of the fact that the biological method for the estimation of vitamin A is not only tedious and expensive, but is open to errors of a rather disturbing magnitude, the need for a more satisfactory method has always been keenly felt. In 1925 Rosenheim and Drummond described the $AsCl_3$ test as the basis of a quantitative method of assay, but the method was greatly improved by the use of $SbCl_3$ and other modifications introduced by Carr and Price [1926]. Drummond and his co-workers have presented evidence that these colour reactions are of practical value in the assay of vitamin A in liver oils, and support for this view has been brought forward by Rosenheim and Webster [1926, 1927], Willimott and Wokes [1927] and others. On the other hand some investigators do not regard the reaction as specific for vitamin A. Some of their objections have been dealt with by Drummond and Morton [1929] and have been shown to be based on unsatisfactory evidence, but those raised by Schmidt-Nielsen were, however, more serious, in that superficially it did not appear that the discrepancies could be attributed to obvious differences of technique.

The work recorded in this paper was undertaken for the purpose of examining the claims of Schmidt-Nielsen, and relates to a number of fish-body and -liver oils which have been studied both colorimetrically and biologically. While it was in progress, Norris and Danielson [1929] published data concerning colorimetric and biological tests of the vitamin A values of some Pacific coast salmon-body oils. They concluded that the results of the colorimetric assays agreed within reasonable limits with those obtained from the biological method. Both their colorimetric and biological values (3–10 units, United States Pharmacopœia) are, however, so low that, in our opinion, their significance is not great.

Schmidt-Nielsen [1928, 1, 2, 3] stated that the body oils of sprat, mackerel and herring as well as the liver oil of the basking shark were, according to his biological tests, good sources of vitamin A, but that they did not react with arsenic or antimony trichloride to give the characteristic blue colour. Generally speaking he found that a blue colour was given by the liver oils of bony fishes such as cod, tusk, ling and haddock, whereas the liver oils of most of the Elasmobranchs, sharks and rays, gave only a feeble reaction, the colour quickly changing to reddish or brown. On the other hand, the liver oil of salmon gave an intense blue colour with $SbCl_3$ (150–200 blue units on the Rosenheim-Schuster colorimeter), which apparently bore no relation to its vitamin A value as indicated by the animal tests. These results led him to conclude that there is no strict relation between the colour reaction and the biological assay for vitamin A, and that the blue colour reaction is probably due to substances which sometimes accompany the vitamins.

EXPERIMENTAL.

The body and the liver oils of salmon and herring were prepared in the laboratory by grinding the flesh or the livers with anhydrous Na_2SO_4, and extracting the dry powder with ether in an atmosphere of nitrogen. The ether was removed in an atmosphere of nitrogen, and the oils were sealed in amber glass ampoules in which the air was replaced by the inert gas. Salmon body and liver gave deep orange-red oils, the yield being approximately 11·5 and 7·0 % respectively, whilst herrings gave a yellow body oil and a brownish-red liver oil in yields of approximately 7·6 and 10·0 %. The herring-liver oil had a distinct green fluorescence. Dr Schmidt-Nielsen also very kindly supplied us with samples of herring-body oil and a sample of the liver oil of the basking shark examined by him [Schmidt-Nielsen, 1928, 2; 1929]. These six samples were submitted to a comparative assay of vitamin A by the colorimetric and biological methods.

The colorimetric test was carried out according to Carr and Price's [1926] modification of Rosenheim and Drummond's method [1925], the colours being matched after 30 seconds. In the case of body oils which do not give a perceptible blue colour in 10 % dilution in chloroform, 0·2 cc. of the oil itself was used and is referred to as 100 % dilution in the table. A mean of at least three readings was taken. The original colour of the oil was also measured with the same colorimeter in suitable dilutions.

In the case of oils which show a very deep blue colour with $SbCl_3$, it is often not possible to obtain a satisfactory match at 10 % dilutions. With the Rosenheim-Schuster colorimeter the most reliable readings are usually obtained by matching at dilutions which give 5–8 blue units. As the intensity of the blue colour is not strictly proportional to the degree of dilution, it is necessary to obtain five or six readings at different dilutions and plot an intensity-dilution curve, by reference to which 10 % dilution values can be interpolated for purposes of comparison. The values of the herring and salmon-liver oils were obtained in this manner.

The technique of the biological test employed was as follows.

Young rats at an average weight of 45–50 g. were put on a vitamin A-free basal diet consisting of

Purified caseinogen	...	20 parts
Purified rice starch	...	71 ,,
Salt mixture ...	...	4 ,,
Dried brewer's yeast	...	5 ,,

The antirachitic factor was supplied in the form of irradiated ergosterol prepared in the laboratory. A dose of 0·01 mg. of this preparation dissolved in ethyl oleate was given twice weekly to each rat throughout the preparatory and the testing periods.

The majority of the rats showed a steady weight after 4–5 weeks on the basal diet. They were then given measured supplements of the oils, which were administered directly into the mouth every morning. Ethyl oleate was used as the diluent, and the solutions were kept in brown glass ampoules of small capacity, and stored in a vacuum desiccator in a cold, dark cupboard. Fresh solutions were made every week or 10 days[1].

The tests of the supplements were carried on usually for 3 weeks in the case of rats which showed little or no growth, whilst they were continued for 4–6 weeks if the material under investigation induced appreciable growth. In the former case the supplement was changed to an adequate dose of another oil known to contain vitamin A, in order to demonstrate that the animal was still capable of growing. In a few cases, however, the rats rapidly declined in weight within a few days, with an onset of deficiency symptoms, and died before they could be saved by the administration of vitamin A.

The results of the colorimetric and biological tests are recorded in Tables I and II respectively, and the Figures 1–6 illustrate representative growth curves.

It will be evident from the data there given that the results are, on the whole, reasonably definite. The figures in the last column of Table I show that the $SbCl_3$ reaction is practically negative for the three body oils from salmon and herring, and for Dr Schmidt-Nielsen's preparation of the liver oil of the basking shark. The blue units given by herring-liver oil are of the same order as those of a good average sample of cod-liver oil, while those of the salmon-liver oil are about six times greater.

The results of biological tests are in satisfactory agreement with those of the colorimetric tests.

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[1] The use of ethyl oleate as a diluent for this purpose was thought at first to be a great improvement in technique [Drummond and Morton, 1929]. It has been found unsatisfactory, however, in one respect. Probably owing to the presence of acids more highly unsaturated than oleic, autoxidative changes tend to occur and may involve the substance being tested. These can be prevented by replacing the natural antioxidant in olive oil by a substance such as quinol. In this investigation we guarded against deterioration of the solutions by making them up freshly every week. We have satisfied ourselves that the vitamin D content of the solution of irradiated ergosterol in ethyl oleate does not fall appreciably over a period of several weeks.

Table I. *Colorimetric tests.*

Nature of the oil	Dilution (%)	Colorimetric units of the oil	Colorimetric units with $SbCl_3$ reagent
Salmon-body oil	10	5·0 yellow 4·6 red	Yellowish red tint without any blue
	100	—	0·4 red 0·2 blue 1·5 yellow
Herring-body oil	10	—	Yellowish tint changing to red after 1–2 min.
	100	6·0 yellow 1·0 red	0·2 red 0·5 yellow 0·3 blue Red increasing after 1–2 min.
Herring-body oil (Schmidt-Nielsen)	100	0·6 red 2·8 yellow	0·2 red 0·6 yellow 0·3 blue After standing 2 min. 0·6 red 0·9 yellow 0·3 blue
Basking shark-liver oil (Schmidt-Nielsen)	10	--	0·3 red 0·6 yellow No perceptible blue
	100	0·8 red 4·0 yellow	Forms a white precipitate
Herring-liver oil	10	3·0 red 0·2 blue 0·2 yellow	11·2 blue — yellow
Salmon-liver oil	10	30·0 yellow 7·5 red	59·0 blue 13·6 yellow

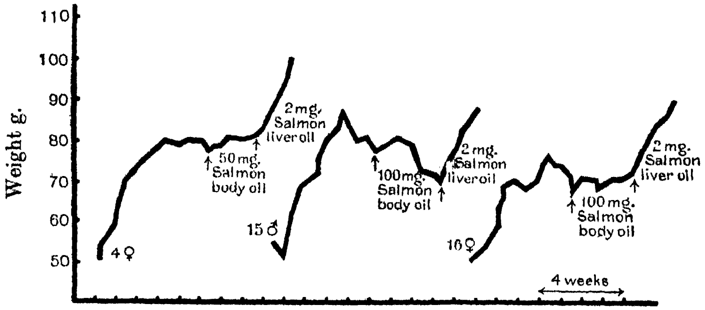

Fig. 1. Salmon-body oil.

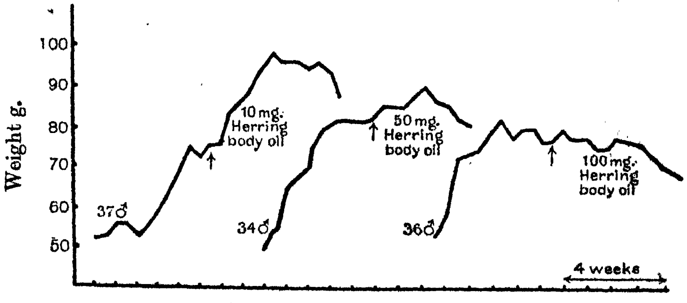

Fig. 2. Herring-body oil.

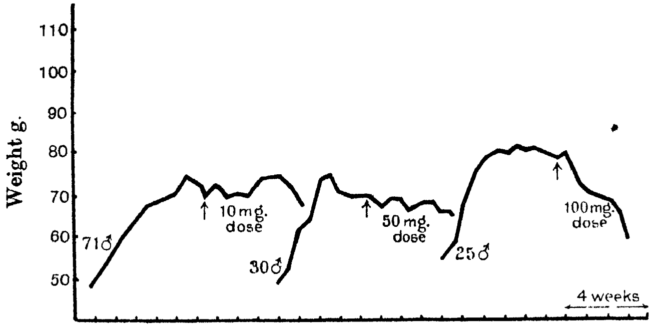

Fig. 3. Herring-body oil (Schmidt-Nielsen).

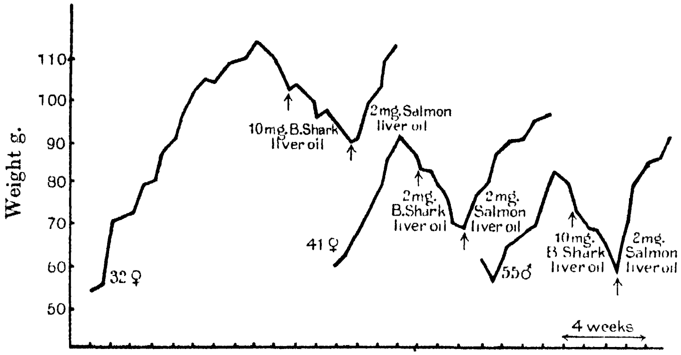

Fig. 4. Basking shark-liver oil (Schmidt-Nielsen).

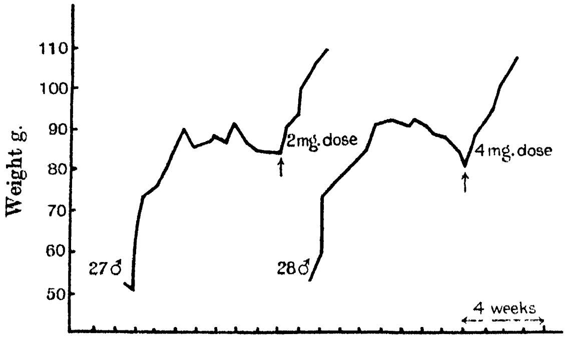

Fig. 5. Herring-liver oil.

Salmon-body oil did not produce any appreciable amount of growth in any of the five rats employed, in doses of 10, 50 or 100 mg. Four of these rats showed a normal response when later the supplements were changed to 2 mg. of salmon-liver fat (Fig. 1). Rat No. 14, however, rapidly declined in weight, had acute ophthalmia of both eyes and died after 13 days. Ophthalmia was also acute in rat No. 10, of a moderate degree in rat No. 4, and only as a translucent film on the cornea of rats No. 15 and No. 16.

Table II. *Biological tests.*

Nature of the oil	Rat No.	Sex	Dose (mg.)	Total time of test (days)	Total change in weight (g.)	Average growth per week (g.)
Salmon-body oil	10	♂	10·0	27	− 4	− 1·0
	14	♂	10·0	13	− 11	− 5·9
	4	♀	50·0	17	+ 4	+ 1·6
	15	♂	100·0	20	− 6	− 2·1
	16	♀	100·0	20	+ 2	+ 0·7
Herring-body oil	37	♂	10·0	42	+ 10	+ 1·6
	42	♂	10·0	6	− 2	− 2·3
	57	♀	10·0	7	− 4	− 4·0
	34	♂	50·0	25	− 1	0·0
	36	♂	100·0	24	0	0·0
Herring-body oil (Schmidt-Nielsen)	33	♂	10·0	67	+ 9	+ 0·9
	56	♀	10·0	10	− 20	− 14·0
	71	♂	10·0	24	− 2	− 0·6
	30	♂	50·0	25	− 5	− 1·4
	25	♂	100·0	22	− 20	− 6·3
Basking shark-liver oil (Schmidt-Nielsen)	36	♂	1·0	10	− 10	− 7·0
	41	♀	2·0	16	− 14	− 6·1
	48	♀	2·0	65	+ 25	+ 2·7
	32	♂	10·0	23	− 10	− 3·0
	44	♀	10·0	10	− 5	− 3·5
	55	♂	10·0	15	− 14	− 6·5
	47	♀	20·0	16	− 8	− 3·5
Herring-liver oil	27	♂	2·0	19	+ 20	+ 7·3
	26	♀	4·0	19	+ 27	+ 9·9
	28	♂	4·0	19	+ 23	+ 8·4
Salmon-liver oil	5	♂	0·3	33	+ 14	+ 3·0
	6	♂	0·5	33	+ 22	+ 4·7
	21	♂	1·0	30	+ 30	+ 7·0
	23	♀	1·0	30	+ 31	+ 7·0
	13	♂	2·0	30	+ 30	+ 7·0
	24	♂	2·0	30	+ 41	+ 9·6
	4	♀	2·0	16	+ 20	+ 8·7
	15	♂	2·0	14	+ 17	+ 8·5
	16	♀	2·0	14	+ 19	+ 9·5
	32	♂	2·0	17	+ 20	+ 8·2
	41	♀	2·0	28	+ 28	+ 7·0

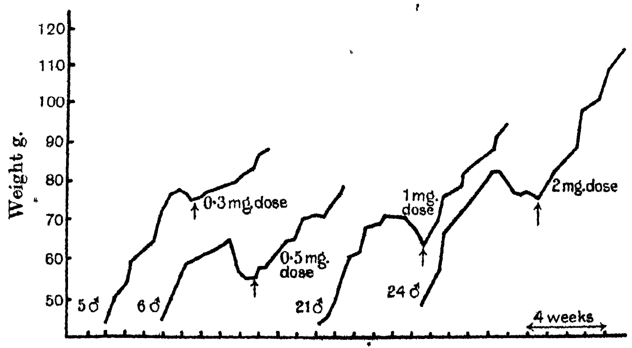

Fig. 6. Salmon-liver oil.

Herring-body oil was also tested in doses of 10, 50 and 100 mg. Some of the test rats showed a slight tendency to grow during the first two weeks, then became steady in weight and finally declined showing ophthalmia and other symptoms of vitamin A deficiency. Only rat No. 37 showed an appreciable degree of growth by increasing 20 g. in the first 20 days of the test. Later its weight declined, and it finally died on the 42nd day, having acute ophthalmia of both eyes during the last week.

Herring-body oil obtained from Dr Schmidt-Nielsen was tested in the same doses as the other herring-body oil, but four of the rats showed no growth and generally rapidly declined in weight. Rat No. 33, however, like the one mentioned in the last paragraph, put on weight in the first 3 weeks, then steadied, declined and died with all the symptoms of vitamin A deficiency. In view of such abnormal cases it is wise to maintain the test for at least 5–6 weeks before conclusions regarding the vitamin A potency are reached. The importance of a long test period has been emphasised by Coward and Key [1928] and Drummond and Morton [1929].

The liver oil of the basking shark was tested in doses of 1, 2, 10 and 20 mg. and gave negative results. Some of the rats after 3 weeks of declining weight were given 2 mg. of salmon-liver oil and promptly showed a very rapid response in growth (Fig. 4).

The herring-liver oil gave normal growth in doses of 2–4 mg. (Fig. 5). The limited quantity of the oil did not permit further tests with smaller doses or for longer periods.

The salmon-liver oil was as potent biologically as was to be expected from its colorimetric reaction. As the tests presented in Table II will show, it gave appreciable growth in doses of 0·3 and 0·5 mg. per day and a very good response in 1·0 and 2·0 mg. doses (Fig. 6).

DISCUSSION.

The biological results are, we think, in reasonably good agreement with the colorimetric tests. The three body oils of herring and salmon, and the liver oil of the basking shark neither gave a positive colorimetric test nor showed activity in the biological tests. An average sample of cod-liver oil which induces normal growth by a dose of 2–4 mg. per day shows colorimetrically about 10 blue units in 10 % dilution. The herring-liver oil with 11·2 blue units is found to be biologically active in a dose of 2 mg. The salmon-liver oil which shows 59·0 blue units is active in 0·3 mg. dose. The results agree with each other as well as can be expected, having regard to the variations to which the biological test is liable [Drummond and Morton, 1929].

The results of the colorimetric tests are similar to those recorded by Schmidt-Nielsen, except that he obtained 150–200 blue units for his sample of salmon-liver oil. In view of the probability that Schmidt-Nielsen refers his units to 20 % dilutions, as was originally proposed by Carr and Price [1926], it must be borne in mind that the intensity of the blue colour is not in strict mathematical relation to the dilution employed. In higher dilutions the curve slopes away from the intensity axis, *i.e.* the observed intensity is lower than the calculated one. This point has also been emphasised by Wokes and Willimott [1927, 1, 2]. If an oil gives 10·0 blue units in 1 % dilution, it will usually give values of the order of 60–65 units in 10 % dilution and not 100 units as would be expected, though the steepness of the curve varies with the individual oils. The apparent disagreement between the two colorimetric tests for salmon-liver oil may arise because Schmidt-Nielsen refers his units to 20 % dilutions and obviously calculates them from a lower dilution.

The biological results of Schmidt-Nielsen deviate very widely from ours. His animal tests led him to assign 50–100 units (U.S.P.) to a number of samples of herring-body oils, and 100 units (U.S.P.) to the sample he so kindly sent us for a comparative test. Our examination of this sample failed to detect the presence of appreciable amounts of vitamin A in doses as high as 100 mg. The sample of the basking shark oil prepared by him in June 1925, and tested biologically in his laboratory in the beginning of 1926, gave him a value of 10,000 units (U.S.P.). Tested again in the middle of 1927, its biological value was only 500–1000 units (U.S.P.), whilst the colour test applied about the same time was negative. This same oil was tested by us in June 1929, and, as reported above, yielded both colorimetrically and biologically negative results. The sample of salmon-body oil tested by Schmidt-Nielsen was active to a degree represented by 20–30 units (U.S.P.) whilst our sample was inactive in doses up to 100 mg. His sample of the salmon-liver oil contained 600 units (U.S.P.) while the test carried out by us indicated an activity of the order of over 3000 units (U.S.P.).

Recently Schmidt-Nielsen [1929] has published additional evidence in support of his view that there is no agreement between the colorimetric and the biological tests. These experiments cover several additional examples, namely, the body oil of halibut and the blubber oil of whale, both showing negative colorimetric tests and 30–40 biological units (U.S.P.): the liver oils of rabbit-fish and the little spinous dog-fish giving 0·2–1·5 blue units and 300 biological units (U.S.P.): and the liver oils of salmon and halibut giving 200 and 700 blue units respectively. In his first experiments Schmidt-Nielsen found the biological activity of these last mentioned oils to be of the same order as the common cod-liver oil, but in this communication he states that on extending his animal experiments to smaller doses they were found to be considerably richer in vitamin A, and one of them (halibut-liver oil) gave as much as 25,000 units (U.S.P.).

Schmidt-Nielsen's results can be grouped into two categories. In the first place there are the oils which, according to him, give a negative colour reaction, but a varying, though appreciable, degree of biological activity. Such are the body oils of herring, sprat, mackerel, halibut and salmon, the blubber oil of whale, and the liver oils of the rabbit-fish, dog-fish, and basking shark. Some of these have only a very small degree of activity, 20–40 units (salmon and halibut-body oils and the blubber oil of whale). Others are rather more active, 50–100 units (herring-body oils), whilst a third group possesses a moderate degree of activity, 300–1000 units (liver oils of the rabbit-fish, dog-fish and basking shark).

The other class consists of the liver oils of certain bony fishes, *e.g.* salmon and halibut, which give a very intense blue colour reaction but show a comparatively low biological activity.

We have confirmed Schmidt-Nielsen's observation that the oils in the first category yield very weak or negative colorimetric tests. It is between the results of his biological tests and ours that the serious discrepancy arises, and it is not a simple matter to arrive at a satisfactory explanation, unless details of the tests employed in the two laboratories are closely compared, especially in regard to the breeding and diet of the stock animals, the length of the test, and range of doses tested, the number of animals employed to test each dose and the variations in response of individual animals. It is important, however, to remember that unless rather large numbers of animals are employed for each test, the results of biological experiments cannot be fairly expressed in such units as those proposed by the United States Pharmacopœia. According to this unfortunately premature method of assay, a 10 mg. dose is equivalent to 100 units and one of 12 mg. to 80 units, if they both induce a weekly growth of the order of 2–4 g. In our experience it is a difficult enough matter in many cases to differentiate between the responses given by 10 and 20 mg. doses, and it is impossible to attach importance to values based on differences which would not bear critical examination.

In view of the observations made in this laboratory on the sensitivity of the biological test [Drummond and Morton, 1929] the values of 20, 30 and 40 units given to some oils by Schmidt-Nielsen have, in our opinion, no real significance.

They can be interpreted as indicating that the oils in question are of low vitamin A value, but it is impossible to regard them in any sense as quantitative.

Actually, we are inclined to attribute many of the apparent discrepancies between results of the biological tests carried out by Schmidt-Nielsen and those obtained by us to his applying the U.S.P. method of evaluation to unsuitable cases, namely those of oils of low vitamin A content.

In view of the errors associated with the colorimetric assay, to which attention has been drawn, and those of very much more serious dimensions that are almost inseparable from the biological test, unless very large numbers of animals are used, it seems unreasonable to seek an exact quantitative relation between the two methods. The nature of the relationship has been considered in a previous study [Drummond and Morton, 1929], and, bearing in mind the limitations of both methods, we consider that the results of the biological and colorimetric tests described in the present paper are in satisfactory agreement.

In the biological tests, cases are sometimes encountered in which an appreciable amount of growth is observed during the first week or two in which the supplement is given. The growth is, in these cases, not maintained and is followed by a rather sharp decline and the sudden onset of deficiency symptoms. Such animals probably carry with them into the testing period a reserve of the vitamin which is not entirely depleted during the preparatory period.

The disagreement between Schmidt-Nielsen's colorimetric and biological tests of the oils in the second category, namely, halibut and salmon-liver oils, is easily explained. It has been shown above that the results of his colorimetric tests, having been calculated from low dilutions, are much higher than their real values. The complete results of his biological experiments are not yet available, but in his later communication referred to above [Schmidt-Nielsen, 1929] he has stated that these oils which he first believed to be of the same order of biological activity as a common cod-liver oil, having now been tested in smaller doses, prove to be much richer in vitamin A than he had expected. In the case of halibut-liver oil he obtained growth in daily doses of 0·04 mg. (25,000 units U.S.P.), which appears to be in good agreement with its colorimetric value.

SUMMARY.

Schmidt-Nielsen, on the basis of a comparative colorimetric and biological assay for vitamin A of a number of fish oils, has claimed that there is no relation between the results obtained by the two methods, and consequently concluded that the blue colour reaction with $AsCl_3$ or $SbCl_3$ is not specific for vitamin A.

Six samples of fish-body and -liver oils, including two samples kindly supplied by Dr Schmidt-Nielsen, have been examined both colorimetrically and biologically for vitamin A. The results of animal tests agree within reasonable limits with those of the colorimetric method, although we make no claim that the colour reaction is specific.

The disagreement between Schmidt-Nielsen's results and ours is due possibly to differences of technique, and to his attempting to express the results of the biological test, which is liable to wide variations, in terms of the units proposed by the United States Pharmacopœia.

This is especially liable to lead to erroneous results when oils of low vitamin A content are examined.

We wish to express our indebtedness to the Medical Research Council for a grant from which the expenses of this investigation were defrayed.

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V. THE PRESERVATION OF THE ANTISCORBUTIC VITAMIN IN LEMON JUICE.

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I. The preservative action of potassium metabisulphite and lemon rind oil.

The first work on the preservation of the antiscorbutic vitamin was performed by Davey [1921], who found that lemon juice could be preserved satisfactorily at a low temperature (about 0°) by both potassium metabisulphite and lemon rind oil respectively. At laboratory temperature preservation was unsatisfactory in the case of metabisulphite, but reliable in that of rind oil. At 37°, neither rind oil nor metabisulphite was satisfactory. An interesting result of Davey's work lies in the fact that apparently 0·09 % metabisulphite is less satisfactory in its preservative effects on the antiscorbutic vitamin than is 0·06 % metabisulphite. This suggests that metabisulphite (or actually sulphur dioxide) has a destructive effect on vitamin C. Delf [1925] states that lemon juice containing added metabisulphite retains its flavour well after storage for 4½ years at laboratory temperature, but that in the case of juice containing 0·06 % metabisulphite, only one-sixth of the original antiscorbutic potency remained after this time. Over a period of time, the preservative effects of 0·06 % metabisulphite and lemon rind oil respectively were similar, and were superior to that of 0·09 % metabisulphite.

A survey of the literature on the behaviour of the antiscorbutic vitamin shows that it is relatively stable in acid solutions, but is destroyed rapidly in alkaline solutions in the presence of air [Harden and Zilva, 1918; Hess and Unger, 1919]. Oxidation has a definite destructive action on the potency of active solutions of vitamin C [Hess and Unger, 1921; Zilva, 1921; Dutcher, Harshaw and Hall, 1921]. Zilva [1923] finds that, whilst in the presence of air vitamin C is rapidly destroyed in alkaline solution, in the absence of air destruction is prevented. More recent work by Zilva [1927] shows that the stability of the antiscorbutic vitamin runs parallel with the presence of a reducing factor which he estimates by titration with phenolindophenol in neutral solution. Whilst the vitamin and the reducing factor are two distinct substances, the reducing factor being destroyed more rapidly than vitamin C, he finds that in all cases there is a distinct parallelism between the two substances. In a later paper Zilva [1928] suggests that "the stability of the antiscorbutic factor in lemon juice is conditioned by the presence of a reducing principle and of a factor, the functioning of which is destroyed by heat. ...The reducing property of the solution acted as a 'reduction buffer' for the antiscorbutic vitamin." "Although acidity does not prevent the destruction of this last factor" (the heat-labile) "by heat, it exercises definite protection of the reducing and antiscorbutic principles after anaerobic heating." Szent-Györgyi [1928] suggests that this reducing factor is identical with the hexuronic acid which he isolated from various juices and which plays an important function in peroxidase systems.

The present investigation is an attempt to develop the work initiated by Davey and Delf, and to discover the conditions necessary for satisfactory preservation of vitamin C in lemon juice.

EXPERIMENTAL.

All samples of lemon juice used in this investigation were stored (while in the laboratory) in well-stoppered bottles (previously sterilised in a steamer) at room temperature. The temperature was constant between 15° and 18° during the tests. After storage, the juices were administered in daily doses to young guinea-pigs of approximately 300 g. weight, kept on a basal diet of bran and oats with water *ad libitum*. Vitamins A and D were supplied daily in the form of cod-liver oil (0·05–0·1 g. daily per animal). On this basal diet the guinea-pigs grow well until the onset of scurvy. Immediately before administration, the juice was partly neutralised with calcium carbonate, a procedure which does not involve any destruction of the vitamin as was shown by a blank experiment. During the progress of the experiment, the characteristic symptoms of scurvy were noted and *post-mortem* examinations were carried out on each animal. Throughout this series of experiments the presence or absence of characteristic haemorrhages and beading of rib junctions was taken as the decisive factor in diagnosing scurvy.

As far as possible, the minimum protective dose of juice was used to define antiscorbutic potency. Where no actual determination of the minimum protective dose was made, the time taken to develop scurvy on any particular dose of juice was assumed to give a reasonable idea of the antiscorbutic potency. It is, of course, realised that this latter procedure is only approximate, since guinea-pigs vary considerably in the time taken to show symptoms of scurvy. When, however, three or more guinea-pigs, receiving doses varying between 1·5 and 3·5 cc., show symptoms of acute scurvy after 20–30 days, it is evident that the juice in question contains very little antiscorbutic vitamin.

Comparative effects of potassium metabisulphite and lemon rind oil.

Fresh lemons were cut in half and squeezed by hand on a glass squeezer. The expressed juice was filtered through fine muslin and was then kept in previously sterilised stoppered Winchester quart bottles. The required amounts of potassium metabisulphite and lemon rind oil were added to the respective samples as follows: 0·02 % potassium metabisulphite, 0·07 %, 0·275 %, 0·5 %

Table I. *Preservative action of potassium metabisulphite and lemon rind oil*

Sample	Animal	Sex	Dose (cc.)	Duration of exp. (days)	*Post-mortem* and general conclusions	Minimum protective dose (cc.)	General condition of juice
Lemon juice + 0·02 % metabisulphite	273	♀	1·0	23	Acute scurvy in each case. All the animals had to be killed after showing severe symptoms of scurvy. *Post-mortem* examinations showed extensive haemorrhages	3·5	Juice clear, golden-yellow colour, heavy growth of moulds on surface
	260	♀	1·5	19			
	290	♂	2·0	23			
	286	♀	2·5	25			
	281	♀	3·0	30			
	262	♀	3·5	25			
Lemon juice + 0·07 % rind oil	282	♂	1·0	27	Acute scurvy—severe extensive haemorrhages	2·5	Juice slightly cloudy and slight growth of moulds: much less than juice with metabisulphite
	271	♀	1·5	48	Acute scurvy—severe extensive haemorrhages, femora and costochondral junctions		
	268	♀	2·0	27	Acute scurvy—severe extensive haemorrhages, femora and costochondral junctions		
	279	♀	2·5	48	Slight haemorrhage on jaws: otherwise quite normal and healthy		
	265	♀	3·0	48	Slight haemorrhage on jaws: otherwise quite normal and healthy		
	283	♂	3·5	48	Abscess under jaw: otherwise quite normal and healthy		
Lemon juice + 0·275 % rind oil	266	♂	1·0	27	Acute scurvy—extensive haemorrhages	3·0–3·5	Juice slightly cloudy: no moulds
	274	♀	1·5	27	Acute scurvy—extensive haemorrhages		
	258	♀	2·0	48	Scurvy—severe haemorrhages, femora, etc.		
	259	♀	2·5	27	Acute scurvy—extensive haemorrhages		
	285	♀	3·0	48	Slight haemorrhages on jaws: otherwise quite healthy		
	269	♂	3·5	48	Spot of haemorrhage on femora and jaws: otherwise quite healthy		
Lemon juice + 0·5 % rind oil	267	♀	1·0	19	Acute scurvy—extensive haemorrhages	3·0–3·5	Juice clear: no moulds
	276	♀	1·5	40	Acute scurvy—extensive haemorrhages		
	280	♂	2·0	48	Scurvy		
	264	♀	2·5	23	Acute scurvy—extensive haemorrhages		
	278	♂	3·0	48	Haemorrhage on jaw and costochondral joint (slight)		
	275	♂	3·5	48	Slight haemorrhage on jaw: otherwise quite healthy		
Lemon juice + 1·1 % rind oil	268	♀	1·0	35	Acute scurvy—extensive haemorrhages	3·5	Juice clear: no moulds
	277	♀	1·5	39	Scurvy—slight haemorrhages, femora and costochondral junctions		
	289	♀	2·0	39	Scurvy—severe femoral haemorrhages		
	257	♂	2·5	39	Scurvy—severe femoral haemorrhages		
	261	♀	3·0	39	Scurvy—severe haemorrhages		
	284	♀	3·5	39	Severe haemorrhages, one femur, otherwise healthy		

and 1·1 % lemon rind oil. After storage for 2½ months at room temperature the juices were administered to young guinea-pigs in daily doses ranging from 1·0 cc. to 3·5 cc. (Table I).

The results in Table I indicate that lemon rind oil is a better preservative than 0·02 % potassium metabisulphite from the point of view of gross fermentation and mould growth as well as from that of antiscorbutic potency. Moreover, it is seen that although the juices preserved with 0·275 % rind oil and above are better preserved in the gross sense, they are if anything slightly less potent in vitamin C than the juice preserved with 0·07 % rind oil, *i.e.* one-sixteenth of the proportion of rind oil to juice normally present in the average fresh lemon.

Following on this, samples of lemon juice were obtained from Sicily, preserved with 0·04 % potassium metabisulphite and 0·07 % and 0·275 % rind oil to test the relative preservative action of rind oil and metabisulphite under commercial conditions. In addition, a further sample was sent over in a cask which had been fumigated with a weak sulphur fume (SO_2) immediately before the juice was introduced into the cask. This last sample contained no other preservative. With the exception of the juice preserved with 0·04 % potassium metabisulphite, all the samples had fermented as was evidenced by the escape of gas when the samples were opened. It is to be noted that 0·04 % metabisulphite is a satisfactory preservative against gross fermentation, whereas 0·02 % metabisulphite, as used in the previous experiment, is unsatisfactory. Table II summarises the condition of the various samples of juices on arrival.

Table II. *Condition of preserved juices.*

Sample of juice	Treatment	Condition
A	Preserved with 0·04 % metabisulphite	No fermentation: juice clear
B	„ „ 0·07 % rind oil	Juice cloudy: some fermentation
C	„ „ 0·275 % „	„ „
D	Cask sulphured	Juice cloudy and fermented

As preservatives against fermentation, rind oil and mere sulphuring of the cask are definitely inferior to 0·04 % potassium metabisulphite. Only in the case of 0·04 % potassium metabisulphite was a palatable juice obtained.

From the date of pressing until the commencement of the experiment about a month elapsed. During this time the juices were exposed to the treatment ordinarily undergone by commercial imported lemon juice. Doses ranging from 1·0 cc. to 3·0 cc. each of the four samples were administered to the test guinea-pigs (Table III).

From these results it will be seen that the findings of the laboratory experiment are confirmed under commercial conditions with respect to the effect of metabisulphite and rind oil on the antiscorbutic vitamin. Here again the juice preserved with 0·07 % rind oil appears to be slightly more potent in vitamin C than that preserved with 0·275 % rind oil. An interesting fact is found in connection with the potency of the sample kept in a cask which had merely been fumigated with sulphur dioxide. Here the concentration of sulphur dioxide was such as to be practically undetectable. This juice had preserved a high proportion of its antiscorbutic potency, in contradistinction to the sample preserved with 0·04 % potassium metabisulphite. These facts show definitely that potassium metabisulphite tends to destroy the antiscorbutic potency of lemon juice; if not directly, by reacting with some factor on which the stability of vitamin C depends. That this latter explanation is probably the case will, it is hoped, be shown in a later paper when the effect of potassium metabisulphite on the loss of reducing power of lemon juice is reported.

Table III. *Preservative action of potassium metabisulphite and lemon rind oil.*

Sample	Animal	Sex	Dose (cc.)	Duration of exp. (days)	*Post-mortem* and general conclusions	Minimum protective dose (cc.)
A. Lemon juice + 0·04 % metabisulphite	395	♀	1·0	52	Acute scurvy—extensive haemorrhages	> 3·0
	329	♀	1·5	43	Acute scurvy—extensive haemorrhages	
	312	♂	2·0	60	Acute scurvy—extensive haemorrhages	
	320	♀	2·5	46	Acute scurvy—extensive haemorrhages	
	318	♂	3·0	74	Acute scurvy—extensive haemorrhages	
B. Lemon juice + 0·07 % rind oil	340	♀	1·0	24	Acute scurvy—extensive haemorrhages	2·0–2·5
	345	♀	1·5	98	Slight scurvy—slight haemorrhages, femur	
	347	♂	2·0	98	Very slight beading of rib junction. No haemorrhages	
	324	♀	2·5	98	Quite normal and healthy	
	327	♂	3·0	98	Quite normal and healthy	
C. Lemon juice + 0·275 % rind oil	351	♀	1·0	30	Scurvy—slight haemorrhages	2·5
	349	♂	1·5	99	Slight scurvy—slight haemorrhages	
	343	♀	2·0	87	Slight scurvy—liver and kidney diseased	
	346	♂	2·5	99	Quite normal and healthy	
	341	♀	3·0	99	Quite normal and healthy	
D. Lemon juice in sulphured cask	350	♂	1·0	98	Slight scurvy—slight haemorrhages	2·0–2·5
	333	♀	1·5	91	Slight scurvy—haemorrhages, femora, etc.	
	322	♀	2·0	98	Very slight scurvy—very slight haemorrhages	
	310	♀	2·5	98	Quite healthy	
	342	♀	3·0	98	Quite healthy	

It has been stated that, except in the case of the juice preserved with 0·04 % potassium metabisulphite, the samples of imported lemon juice had fermented and were rather unpalatable. Davey [1921] and Zilva [1924] have stated previously that the sugar in lemon juice may all be fermented without any appreciable destruction of the antiscorbutic fraction. This is the case in the samples of juice used in this experiment. Whilst potassium metabisulphite (or sulphur dioxide) is undoubtedly the best preservative of lemon juice from the point of view of fermentation, it tends to destroy the antiscorbutic vitamin.

The rate of loss of antiscorbutic potency of lemon juice preserved with 0·04 % *potassium metabisulphite.*

It has been shown above that at laboratory temperature, lemon juice preserved with 0·04 % potassium metabisulphite for approximately 1 month had lost at least 50 % of its antiscorbutic potency. It is of interest to determine how quickly the antiscorbutic potency of lemon juice preserved with metabisulphite is lost.

A three weeks' supply of lemon juice was pressed weekly, the pulp being removed by filtration through muslin. The requisite amount of potassium metabisulphite was added. Three lots of guinea-pigs were taken for this experiment and daily doses of 1·5, 3·0, and 4·5 cc. were given. At the end of the first week, the juice was administered to one lot of guinea-pigs and continued for a week. They received juice which had been preserved an average of 10–11 days. At the end of three weeks the second set of guinea-pigs received the juice; this was given for 1 week so that the juice they received had been preserved an average of 25 days. The third set of guinea-pigs received the juice after 5 weeks; this juice was preserved for an average of 39 days. By this means the loss of vitamin C potency in the juice was followed over a period of 39 days (Table IV).

Table IV.

Juice	Animal	Sex	Dose (cc.)	Duration of exp. (days)	*Post-mortem* and general conclusions
Preserved 11 days	451	♀	1·5	96	No scurvy—healthy
	452	♀	3·0	96	No scurvy—perfectly healthy
	453	♀	4·5	96	No scurvy—perfectly healthy
Preserved 25 days	455	♂	1·5	36	Acute scurvy—severe extensive haemorrhages and beading of rib junctions
	454	♂	3·0	72	Acute scurvy—severe extensive haemorrhages and beading of rib junctions
	456	♀	4·5	94	No scurvy haemorrhages: very slight beading of rib junctions: apart from digestive disorders quite healthy
Preserved 39 days	460	♀	1·5	24	Severe extensive haemorrhages and beading of rib junctions Acute scurvy
	462	♀	3·0	29	
	461	♀	4·5	29	

From Table IV it will be definitely seen that the loss of antiscorbutic potency of lemon juice preserved with 0·04 % metabisulphite proceeds with a measurable velocity. It is hoped to demonstrate in a later paper that this loss of vitamin potency is to a certain extent parallel with the loss in reducing power of lemon juice preserved under identical conditions. The fact that vitamin potency and reducing power are present together has been noted previously by Zilva [1927].

II. The effect of acidity on the preservation of vitamin C.

It is a generally established fact that vitamin C is more stable in acid solutions than in alkaline solutions [cf. Zilva, 1923]. Moreover, high acidity also inhibits the growth of most bacteria and moulds. It was thought, therefore, that an addition of acid to lemon juice would serve as a preservative both for the antiscorbutic vitamin and also against gross fermentation. With this end in view, a sample of juice made up to p_H 1·6 with hydrochloric acid was imported from Sicily. On its arrival (about 1 month after pressing) the juice was examined and fed to test guinea-pigs (Table V).

Table V. *The preservative action of hydrochloric acid in lemon juice.*

Sample	Animal	Sex	Dose (cc.)	Duration of exp. (days)	*Post-mortem* and general conclusions
Lemon juice + 0·3 % HCl (p_H 1·6)	316	♂	1·0	92	No apparent scurvy—slight haemorrhages on jaw, otherwise healthy
	335	♀	1·5	92	No apparent scurvy—slight haemorrhages on jaw, otherwise healthy
	308	♀	2·0	92	No apparent scurvy—slight haemorrhages on jaw, otherwise healthy
	311	♂	2·5	92	Quite normal and healthy
	319	♂	3·0	92	Quite normal and healthy

From this table, lemon juice preserved for 1 month with 0·3 % HCl (p_H 1·6) was as potent as fresh lemon juice as an antiscorbutic. It was not preserved in the gross sense however. The juice on arrival was yellow, cloudy and fermented. This sample of juice was tested for its antiscorbutic potency after a further 12 months' storage and was found to have preserved its potency intact, 1·5 cc. being a protective dose for a guinea-pig.

It was next deemed desirable to ascertain whether there was an optimum acidity for the stability of the antiscorbutic vitamin, when kept under ordinary laboratory conditions, and whether the acidity of the juice could be increased still further to inhibit mould growth and fermentation, at the same time maintaining a palatable juice rich in the antiscorbutic vitamin.

Fresh lemons were squeezed and the juice obtained as described above. Samples were adjusted to p_H 1·8, 1·4, 1·0 and 0·6 with pure HCl, whilst one sample was made up to p_H 3·0 with alkali. These samples together with a sample of ordinary (unpreserved) lemon juice were kept at room temperature (15°–18°) for 7–8 months in sterilised bottles, at the end of which time they were fed in doses corresponding to 1·0–3·0 cc. to young guinea-pigs. Immediately before administration to the test animals, the various samples were brought to the same p_H, namely that of fresh lemon juice (p_H 2·2) with standard alkali (or acid in the case of the less acid juice), the dilution of the juice being allowed for in the calculation of the size of dosage (Table VI).

It is apparent from Table VI that vitamin C is destroyed both in the more acid solutions above p_H 1·8 and in the solution at p_H 3·0, though in the last case to a much smaller extent. In the solutions of p_H 1·4 and less it appears that the vitamin is completely absent.

On keeping these solutions it was noted that in addition to the precipitate of pulpy material which invariably shows itself in solutions of lemon juice, a more flocculent precipitate appeared. The bulk of this precipitate increased as the

Table VI. *The effect of acidity on the stability of vitamin C.*

Juice	Animal	Sex	Dose (cc.)	Duration of exp. (days)	*Post-mortem* and general conclusions	Minimum protective dose (cc.)	Condition of juice
Lemon	366	♂	1·5	23	Extensive and severe haemorrhages—acute scurvy	> 3·0	No mould growth or fermentation: the juices turned brown on keeping, possibly due to hydrolysis of the protein in the juice
juice +	371	♂	2·0	23	Extensive and severe haemorrhages—acute scurvy		
HCl	365	♂	2·5	23	Extensive and severe haemorrhages—acute scurvy		
p_H 0·6	368	♂	3·0	23	Extensive and severe haemorrhages—acute scurvy		
Lemon	362	♂	1·5	27	Severe haemorrhages—scurvy	> 3·0	No mould growth or fermentation: the juices turned brown on keeping, possibly due to hydrolysis of the protein in the juice
juice +	373	♂	2·0	23	Severe haemorrhages—acute scurvy		
HCl	378	♀	2·5	21	Severe haemorrhages—acute scurvy		
p_H 1·0	364	♀	3·0	25	Found dead—emaciated condition: slight beading of costochondral junctions, and haemorrhages—incipient scurvy		
Lemon	372	♂	1·5	21	Weak condition—no definite evidence of scurvy	> 3·0	No mould growth or fermentation: the juices turned brown on keeping, possibly due to hydrolysis of the protein in the juice
juice +	382	♂	2·0	24	Found dead: emaciated condition—very slight scurvy		
HCl	379	♂	2·5	21	Severe haemorrhages—acute scurvy		
p_H 1·4	376	♀	3·0	26	Severe haemorrhages—acute scurvy		
Lemon	386	♀	1·5	55	Killed. Fractured femur—no symptoms of scurvy	1·5–2	Slight growth of mould and darkening of juice
juice +	385	♀	2·0	90	Perfectly healthy—no scurvy symptoms		
HCl	369	♀	2·5	90	Perfectly healthy—no scurvy symptoms		
p_H 1·8	367	♀	3·0	90	Perfectly healthy—no scurvy symptoms		
Lemon juice	384	♀	1·5	76	Slight haemorrhage, costochondral junctions—no other scurvy symptoms	1·5–2	Heavy mould and alcoholic fermentation
with no HCl	383	♂	2·0	76	Slight haemorrhage, jaw—no other scurvy symptoms		
p_H 2·2	370	♀	2·5	76	Slight haemorrhage, jaw—no other scurvy symptoms		
	363	♂	3·0	62	Found dead: very emaciated condition—no symptoms of scurvy		
Lemon	374	♂	1·5	67	Extensive haemorrhages—acute scurvy	3·0	Heavy mould and alcoholic fermentation
juice +	389	♂	2·0	75	Found dead: very emaciated—no definite scurvy symptoms		
NaOH	380	♂	2·5	82	Severe haemorrhages—acute scurvy		
p_H 3·0	388	♀	3·0	84	No scurvy symptoms		

latter solutions developed a dark brown colour, the depth of which also varied with increase in acidity. This is possibly evidence of hydrolysis of the protein and carbohydrate of the juice. In these latter solutions no evidence of mould growth or fermentation was detected, whereas in the solutions at p_H 1·8 and 2·2 a slight growth of mould was noted. No great degree of discoloration took place in these two solutions. In the juice adjusted to p_H 3·0 mould growth and strong evidence of alcoholic fermentation were noted. The main result of this experiment is that the antiscorbutic vitamin system is shown to be most stable in the region of the natural p_H of lemon juice, *i.e.* p_H 1·8–2·2.

To test whether any precipitation of the vitamin took place in the more acid solutions of juice (p_H 1·4 and above), the residues of these juices were made up with distilled water to a total volume which was $\frac{2}{3}$ of the original juice preserved. Immediately before administering to test animals, the samples were shaken up well to ensure perfect mixing. 10 cc. of each sample were taken and brought to the normal p_H of lemon juice, the final volume being in each case 20 cc. Daily doses of 2·0 cc., 4·0 cc. and 6·0 cc., corresponding to 1·5, 3·0, 4·5 cc. of the original juice were given, the usual technique being followed. Precautions were taken to ensure that representative samples were given to the test animals, by mixing between each dosage (Table VII).

Table VII. *Residues of juice from experiments in Table VI.*

Sample of juice	Animal	Sex	Dose (cc.)	Duration of exp. (days)	*Post-mortem* and general conclusions
Residue from juice + HCl p_H 0·6	429	♀	1·5	19	All these guinea-pigs were found to show severe extensive haemorrhages and beading of costochondral junctions. Acute scurvy
	436	♂	3·0	19	
	424	♂	4·5	22	
Residue from juice + HCl p_H 1·0	427	♀	1·5	19	
	435	♂	3·0	22	
	423	♀	4·5	19	
Residue from juice + HCl p_H 1·4	434	♂	1·5	22	
	437	♂	3·0	22	
	428	♀	4·5	19	

From this it will be seen that precipitation of the antiscorbutic vitamin does not take place in acid solution but that complete destruction of this factor occurs.

III. The effect of other preservatives on the antiscorbutic potency of lemon juice.

Formic acid and sodium benzoate.

Two samples of lemon juice, preserved with 0·05 % sodium benzoate and 0·25 % formic acid, respectively, were obtained from Sicily. On arrival in the laboratory both of these samples had fermented slightly as was evidenced by the escape of gas when the samples were opened. It was apparent that the preservation against gross fermentation exhibited by these two acids is inferior to that by similar amounts of sulphur dioxide in the form of potassium metabisulphite. Moreover, the palatability of the juices preserved by sulphur dioxide was superior to that of those preserved by sodium benzoate and formic acid.

From the time of pressing until the commencement of the animal feeding tests about a month elapsed. Doses ranging from 1·0 cc. to 3·0 cc. of each of the two samples were administered to the test guinea-pigs (Table VIII).

Table VIII. *Preservative action of sodium benzoate and of formic acid.*

Sample	Animal	Sex	Dose (cc.)	Duration of exp. (days)	*Post-mortem* and general conclusions
Lemon juice + 0·05 % sodium benzoate	332	♂	1·0	50	Slight femoral haemorrhages, sub-maxillary and jaw more severe—scurvy
	334	♀	1·5	50	Severe haemorrhages—acute scurvy
	344	♂	2·0	74	Extensive haemorrhages—acute scurvy
	309	♀	2·5	88	Found dead: haemorrhages and emaciated condition—scurvy
	321	♀	3·0	74	Extensive haemorrhages—acute scurvy
Lemon juice + 0·25 % formic acid	307	♂	1·0	43	Extensive haemorrhages—acute scurvy
	328	♂	1·5	57	Extensive haemorrhages—acute scurvy
	326	♂	2·0	60	Extensive haemorrhages—acute scurvy
	317	♂	2·5	86	Haemorrhages—slight scurvy
	314	♂	3·0	96	Haemorrhages—incipient scurvy

It will be seen that 0·05 % benzoic acid and 0·25 % formic acid are as inefficient as 0·04 % metabisulphite as preservatives of the antiscorbutic vitamin. Furthermore, they keep the juice neither palatable nor of good colour.

Essential oil of cloves.

Essential oils, such as oil of cloves, which have mild antiseptic properties, have been used for the preservation of wine and other substances which suffer deterioration from fermentation. A sample of juice pressed in the laboratory and preserved with 0·1 % oil of cloves was kept for 4–5 weeks at room temperature. No signs of mould growth or fermentation could be detected and the sample of juice was palatable and of good colour (Table IX).

Table IX. *Preservative action of oil of cloves.*

Sample	Animal	Sex	Dose (cc.)	Duration of exp. (days)	*Post-mortem* and general conclusions
Juice + 0·1 % oil of cloves	419	♀	1·5	23	Extensive haemorrhages and beading of rib junctions—acute scurvy
	412	♂	2·0	23	Extensive haemorrhages and beading of rib junctions—acute scurvy
	421	♀	2·5	23	Femoral haemorrhages: slight beading of rib junctions—scurvy
	422	♀	3·0	23	Femoral haemorrhages: slight beading of rib junctions—scurvy

It will be seen that a daily dose of 3·0 cc. of lemon juice preserved with 0·1 % oil of cloves is insufficient to protect against scurvy over 23 days. In fact, it appears that the antiscorbutic potency is lost as completely as with potassium metabisulphite

Glucose and sucrose.

Since sugars and syrups are employed in the preparation of concentrated and dried fruit juice, it appeared advisable to test whether sucrose and glucose acted as preservatives for vitamin C in fresh lemon juice.

Small samples (400 cc.) were placed aside in March 1928, preserved with 30 % glucose, 10 %, 20 %, 30 % and 50 % sucrose.

In February 1929, *i.e.* 11 months after storage, the appearance of the juices was noted (see Table X).

Table X.

Sample	Appearance	Remarks
Juice + 30 % glucose	Slight mould	All the samples smelt as if slightly fermented and there was a slight gas evolution
Juice + 10 % sucrose	Slight mould	
Juice + 20 % sucrose	Slight mould	
Juice + 30 % sucrose	No mould	
Juice + 50 % sucrose	No mould	

Daily doses of 1·5 cc. and 3·0 cc. of three of these samples, lemon juices preserved with 30 % glucose, 10 %, and 30 % sucrose respectively were fed to experimental guinea-pigs, the usual technique being followed (Table XI).

Table XI. *Preservative action of glucose and sucrose respectively.*

Juice	Animal	Sex	Dose (cc.)	Duration of expt. (days)	*Post-mortem* and general conclusions
Lemon juice + 10 % sucrose	445	♀	1·5	24	Scurvy. Femoral and costochondral haemorrhages and beading
	447	♂	3·0	21	Acute scurvy. Severe extensive haemorrhages
Lemon juice + 30 % sucrose	448	♀	1·5	19	Acute scurvy. Femoral haemorrhages and beading of costochondral junctions
	449	♀	3·0	19	Acute scurvy. Femoral haemorrhages and beading of costochondral junctions
Lemon juice + 30 % glucose	444	♀	1·5	26	Very small haemorrhages, femur and costochondral junctions: no beading or other scurvy symptoms. Emaciated condition
	446	♀	3·0	48	No characteristic scurvy, haemorrhages or beading. Very emaciated condition

Judging from the condition of the samples of lemon juice, the various concentrations of sugar were sufficient to inhibit to a great extent the growth of bacteria and moulds. This is in virtue of the osmotic effect. When the antiscorbutic potency of the juices is compared an interesting difference between the behaviour of the two sugars, sucrose and glucose, is noted.

The juices preserved with sucrose had lost all their antiscorbutic potency, the test animals having developed acute scurvy within 3 weeks. Unfortunately the test of the juice containing glucose could not be carried to a conclusive point as the animals became emaciated and suffered from digestive troubles, probably as the result of the high concentration of glucose. Whilst it cannot be said definitely that glucose is a good preservative for the antiscorbutic vitamin, it is obvious that there is an essential difference between the reducing sugar, glucose, and the non-reducing sugar, sucrose, in connection with

Table XII. *Summary of results.*

Preservative	Minimum protective dose	General condition of juice
0·02 % potassium metabisulphite	> 3·5 cc. after 2½ months' preservation	Clear yellow colour: heavy growth of moulds on surface
0·04 % potassium metabisulphite	> 3·0 cc. after 1 month's preservation	Clear: no fermentation
Juice kept in sulphured cask	2·0–2·5 cc. after 1 month's preservation	Cloudy and fermented
0·07 % lemon rind oil	2·5 cc. after 2½ months' preservation	Slightly cloudy: slight growth of moulds
0·275 % lemon rind oil	3·0–3·5 cc after 2½ months' preservation	Slightly cloudy: no moulds
0·5 % lemon rind oil	3·0–3·5 cc. after 2½ months' preservation	Clear: no moulds
1·1 % lemon rind oil	3·5 cc. after 2½ months' preservation	Clear: no moulds
0·07 % lemon rind oil	2·0–2·5 cc. after 1 month's preservation	Cloudy: some fermentation
0·275 % lemon rind oil	2·5 cc. after 1 month's preservation	Cloudy: some fermentation
Hydrochloric acid (p_H 1·6)	1·5 cc. after 1 month's preservation	Yellow, cloudy and fermented
Hydrochloric acid (p_H 1·6)	1·5 cc. after 14 months' preservation	Badly fermented
Hydrochloric acid (p_H 0·6) Hydrochloric acid (p_H 1·0) Hydrochloric acid (p_H 1·4)	> 3·0 cc. after 7–8 months' preservation	No mould growth or fermentation. The juice turned brown, possibly due to hydrolysis of protein in the juice
Hydrochloric acid (p_H 1·8)	1·5–2·0 cc. after 7–8 months' preservation	Slight growth of mould and darkening of juice
Unpreserved lemon juice (p_H 2·2)	1·5–2·0 cc. after 7–8 months' preservation	Heavy mould growth and alcoholic fermentation
Sodium hydroxide (p_H 3·0)	3·0 cc. after 7–8 months' preservation	Heavy mould growth and alcoholic fermentation
0·05 % sodium benzoate	> 3·0 cc. after 1 month's preservation	Brown, cloudy and fermented
0·25 % formic acid	> 3·0 cc. after 1 month's preservation	Brown, cloudy and fermented
0·1 % oil of cloves	> 3·0 cc. after 1 month's preservation	Palatable and good colour; no fermentation
10 % sucrose	> 3·0 cc. after 11 months' preservation	Slight mould growth and fermentation
30 % sucrose	> 3·0 cc. after 11 months' preservation	No mould growth; slight fermentation
30 % glucose	< 3·0 cc. after 11 months' preservation	Slight mould growth and fermentation

vitamin C. The animal receiving 1·5 cc. of the juice containing glucose showed no definite signs of scurvy after 26 days, while that receiving 3·0 cc. showed no symptoms after 48 days. It may be gathered therefore that this sample of juice retained a fair amount of its antiscorbutic potency, but no definite conclusions can be drawn.

DISCUSSION.

Table XII summarises the results on the effect of various preservatives on lemon juice given in the present paper.

These results are very striking. They indicate that those substances which exert the strongest preservative effect against gross fermentation possess the greatest destructive action on the antiscorbutic vitamin system. The juice which contained no added preservative retained its antiscorbutic potency to a great extent. The results with acid are particularly significant, since they indicate that the antiscorbutic vitamin is extremely unstable under conditions which are detrimental to the development of moulds and bacteria.

SUMMARY.

1. Potassium metabisulphite, whilst being the best preservative for lemon juice against fermentation, has a definite destructive action on the antiscorbutic vitamin at laboratory temperature (15°–18°). This destruction proceeds with a measurable velocity.

2. Lemon rind oil, whilst not preserving lemon juice satisfactorily against fermentation at ordinary temperature (15°–18°), is less destructive towards vitamin C than potassium metabisulphite. A concentration of 0·07 % rind oil preserves the vitamin potency slightly better than 0·275 % rind oil, indicating that rind oil has a slight destructive action on the vitamin system.

3. The optimal zone of stability of the antiscorbutic vitamin in lemon juice at ordinary temperature lies between p_H 1·6–2·2, that is, in the neighbourhood of the natural acidity of lemon juice.

4. Lemon juice adjusted with HCl to p_H 1·6 preserves its antiscorbutic activity for 14 months at ordinary temperatures.

5. Sodium benzoate and formic acid exert a destructive action on vitamin C.

6. Oil of cloves exerts a destructive action on the antiscorbutic vitamin comparable with that of 0·04 % potassium metabisulphite.

7. Sucrose fails to preserve vitamin C in lemon juice. Indications are given that glucose may exert a preservative action on the vitamin.

8. It is pointed out that the antiscorbutic vitamin system is particularly unstable under conditions detrimental to the development of moulds and bacteria.

The authors wish to thank the Directors of Messrs J. and J. Colman, Ltd., for permission to publish the results contained in this paper. Their thanks are also due to Miss E. G. Robotham for her careful supervision and feeding of the test animals.

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VI. THE PREPARATION OF GLUTATHIONE FROM YEAST AND LIVER.

By NORMAN WINGATE PIRIE.

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(*Received December 2nd, 1929.*)

Various workers have published modifications of the original Hopkins [1921] method for the preparation of glutathione without increasing markedly either the ease of the method or the purity of the final product. Hopkins [1929] obtained pure crystalline glutathione by a very much simplified technique in which the precipitation of glutathione as its crystalline cuprous salt was the most important feature. The still more simple method now to be described depends entirely for its success on the extreme specificity of this cuprous oxide precipitation in acid solution.

The yield of glutathione by the Hopkins [1929] method is very variable although always large; it appeared that the yield became smaller when large (½ cwt.) quantities of yeast were used than when only a few kilograms were worked up at a time. This loss seemed to be due to the difficulty of washing a large mercuric sulphide precipitate so as to remove all the adsorbed glutathione. An attempt was therefore made to dispense with the precipitation with mercuric sulphate.

The extract obtained on boiling yeast in water was found to give an unsatisfactory cuprous salt when cuprous oxide was added directly to the acidified filtrate. Treatment with quantities of alcohol varying from 2000 to 150 cc. per kg. of pressed yeast was also unsatisfactory, although a filtrate rich in glutathione, as estimated by the method of Perlzweig and Delrue [1927], was obtained. The filtrates obtained from mixtures of pressed yeast with common salt, ammonium sulphate or small quantities of 30 % sulphuric acid contained very little glutathione.

It was observed that the addition of a little ether to pressed yeast resulted in the formation of a smooth cream after a few hours' standing. This was very difficult to filter but filtration was much improved by the addition of sulphuric acid; acid has the further advantage of diminishing autoxidation in the filtrate. In the final method adopted a mixture of alcohol, ether and sulphuric acid is used, the concentration of sulphuric acid must be high both on account of the great buffering power of yeast and also to prevent frothing which occurs in mixtures of yeast, alcohol, ether and insufficient sulphuric acid. This mixture filters somewhat slowly but centrifuges easily and where the latter method of treatment is possible it is to be recommended. By this method the preparation of glutathione is remarkably easy and, using small quantities of yeast, crystalline glutathione may easily be obtained in 3 days.

Since this work was completed Kendall, McKenzie and Mason [1929] have published a method of preparation in which benzene is used to liberate the glutathione from yeast. I had tried toluene and butyl alcohol, adding 5 cc. to 100 g. of pressed yeast, but abandoned them in favour of ether.

EXPERIMENTAL.

20 cc. of concentrated sulphuric acid are added slowly to 100 cc. of 89 % alcohol, the mixture being kept cool; when cold 80 cc. of ether are added. This mixture is poured on to 2000 g. of pressed baker's yeast, coarsely crumbled, in a large jar. The mixture is stirred for a few minutes till homogeneous and poured on to two large Büchner funnels (it is necessary to put kieselguhr on the papers to get clear filtration). Filtration is somewhat slow and after about 4 hours nearly stops, at this point it is best to pour the fluid upper layer on to another funnel and allow the cake to suck dry for a few hours more. Too high a vacuum should not be maintained in the filter flask at first since the filtrate froths as the ether boils off.

The combined filtrate amounts to about 1100 cc., a sample is titrated with sodium hydroxide using Congo red as indicator (the filtrate is generally just acid to Congo red) and the amount of 20 % sulphuric acid necessary to bring the filtrate to 0·5 *N* is added.

Precipitation with cuprous oxide is carried out as in Hopkins's method. The reaction proceeds satisfactorily at 20° but is somewhat more rapid at 40°. About 1 g. of cuprous oxide is required and this is most conveniently added in 2 cc. lots when suspended in about 30 cc. water. When about half the cuprous oxide has been added the salt should be allowed to settle and the mother-liquor poured off for further addition of oxide. At a later stage the addition of cuprous oxide will cause the re-solution of some of the cuprous-glutathione compound already formed; when this occurs the cuprous salt may be reprecipitated by blowing air through the solution for about an hour. The precipitate redissolves on very prolonged aeration Although flocculent and amorphous this compound has the normal copper content (17·0 %) and gives crystalline glutathione on decomposition.

A further quantity of glutathione may be extracted from the yeast by 1000 cc. of 1 % (by vol.) sulphuric acid warmed to about 40°. Mixing is most easily effected by hand and the filtrate is treated precisely as before. A third extract prepared in the same way will also give some cuprous salt. The combined extracts from which the cuprous salt has been separated give a copious precipitate with the mercuric sulphate reagent used by Hopkins. This, after removal of the mercury with H_2S, gives a further yield of cuprous salt.

After a preliminary washing on the centrifuge with 0·5 *N* sulphuric acid the cuprous salt must be washed with water till the washings are free from sulphate. This is very laborious if a centrifuge is used since the washing must be repeated about ten times. Washing may be carried out by filtration if the surface at which filtration is occurring is situated above instead of below the precipitate to be washed. A convenient device may be made with two Jena sintered glass filters (as some filters of grade 3 allow the precipitate to pass, it is safer to use grade 4) of a suitable size. The cuprous salt is put in one and the other placed upside down on top, a watertight joint being made with a piece of wide rubber tubing. The lower funnel is connected to a reservoir of distilled water about a metre above it and the upper one is attached to a flask and filter pump. Washing is continued till the filtrate is free from sulphate and remains so on shaking the filtration apparatus.

After decomposition with H_2S the filtrate from cuprous sulphide is evaporated to small bulk in a vacuum desiccator at room temperature. If the volume of filtrate is too large for this and it is essential to distil *in vacuo* to concentrate the solution, a good pump should be used and the distillation carried out at about 25°. Hopkins (unpublished work) has shown that a solution of wholly crystalline glutathione is converted in part to a gummy material by a few hours' exposure to a temperature of 40°. Crystallisation is generally spontaneous but should the solution reach a syrupy consistency without the appearance of foci rubbing with a glass rod will start crystallisation.

By this method 2 g. of crystalline glutathione are obtained from 2 kg. yeast. From 25 kg. yeast 31 g. glutathione, of which 29 were perfectly crystalline, were obtained. In this case only 7 g. came from the decomposed mercury precipitate. This probably represents glutathione which was autoxidised during the initial filtration and, when small quantities are used, or if the mixture is centrifuged, the extra yield does not repay the labour involved by mercury precipitation.

The same method has been applied satisfactorily to liver. In the case of rabbit liver, 20 cc. of the alcohol-ether-sulphuric acid mixture are added to 100 g. fresh tissue and the mixture is ground to a smooth paste with a little sand. The filtrate generally requires refiltration through kieselguhr. Pig or ox liver is put through a mincing machine before the addition of the alcohol mixture. To avoid the autolytic formation of free cysteine in liver these preliminary operations are carried out as rapidly as possible and in every case the p_H of the tissue has been lowered, by the sulphuric acid, to about 2, *i.e.* out of the range of peptidase action, within 40 minutes of the death of the animal. None of the liver preparations has given any colour with the Sullivan reagent under conditions such that 0·1 mg. cysteine hydrochloride could be detected clearly in the presence of 20 mg. glutathione. It would appear therefore that, as Bierich and Kalle [1928] have suggested, only traces of free cysteine are present in fresh liver, since it has been shown in this laboratory that cysteine forms a cuprous salt similar to that of glutathione[1].

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[1] Glycylcysteine and glycylcysteine diketopiperazine also form cuprous derivatives. I hope to describe these compounds in a paper at present in preparation.

On addition of the alcohol mixture, minced liver becomes dry and granular. It is therefore squeezed in a press and the extract, amounting to 1080 cc. from 2300 g. of ox liver and 300 cc. of the alcohol mixture, filtered through kieselguhr. The filtrate is now treated exactly as in the case of yeast. Glutathione prepared from liver by this method is not wholly crystalline and the analytical figures have been somewhat low, *e.g.* N = 12·5 %, S = 9·93 %, while the ash is sometimes as high as 2 % (the analyses were made by Mr A. R. Colwell in this laboratory). The product seems however, as mentioned above, to be free from cysteine.

Attempts to isolate glutathione by this method from muscle and blood have not so far given satisfactory results.

SUMMARY.

A simple method for the isolation of glutathione from yeast is given. The yield is greater than by any of the methods described hitherto.

The preparation of glutathione from liver is described.

I am grateful to Sir F. G. Hopkins for his interest in this work.

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VII. DETERMINATION OF VOLATILE SUBSTANCES.

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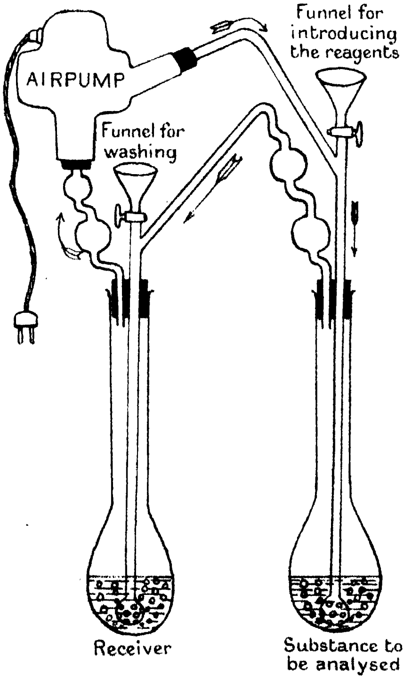
(*Received December 11th, 1929.*)

The quantitative determination of volatile substances in solution cannot be always considered satisfactory for many reasons.

Some methods of measuring the amount of ammonia (Schloesing) require some days for their execution. Folin's method of blowing air through the solution in the cold is attended with some risk of losing a part of the substance with too rapid an air current. The same danger is observed in the measurement of preformed acetone by the formation of iodoform.

Starting from Folin's method, it was decided, in order to eliminate all possibility of loss of the substance to be determined, to enclose the whole system, as is done in Regnault's respiration-chamber. A special apparatus was constructed based on the following principle. Through the liquor to be analysed air is passed, by means of an air-pump, which carries away the volatile substance and enters, in the form of small bubbles, the receiver containing a standard solution in which it is absorbed. After passing through the standard solution the air is again pumped through the liquid by the same air-pump and circulates indefinitely.



In this way the volatile substance, if not absorbed at first in the receiver, is repeatedly brought into contact with the standard solution and is finally completely absorbed. The circulation being effected in a closed system, the possibility of any loss of substance is eliminated.

An experiment with our apparatus was executed on a standard solution of $(NH_4)_2SO_4$.

The amount of ammonia had previously been determined by means of simple distillation with sodium hydroxide and titration. 10 cc. standard solution neutralised 18·7 and 18·8 cc. of $N/10$ acid.

Distilled	Time of distillation (min.)	Amount of $N/10$ H_2SO_4 neutralised
10 cc. of standard solution $(NH_4)_2SO_4$ + 20 cc. 33 % NaOH without heating in our apparatus	(1) 35	18·7
	(2) 20	18·7
	(3) 15	18·7
	(4) 10	18·7

The figures for the determination of ammonia in three different urines by means of our apparatus and the method of Krueger-Reich-Schittenhelm are as follows.

	$N/10$ H_2SO_4 neutralised	
	Krueger method	With our apparatus
25 cc. urine + 10·0 g. NaCl + 1·0 g. Na_2CO_3. Schittenhelm adds some alcohol; we have used vaselin oil	(1) 5·7	5·9 5·6 5·6
	(2) 15·7	15·9 15·7 16·1
	(3) 13·7	13·8 13·8

A series of experiments with varying quantities of alkali and with alcohol (as in the method of Krueger-Reich-Schittenhelm) did not show any advantage. Prolonged distillation (more than 20 minutes) with our apparatus did not increase the amount of H_2SO_4 neutralised.

Excellent results were obtained with the apparatus in the determination of acetone. 2 cc. of a standard solution of acetone in water required 3·8 cc. of $N/10$ iodine. From the same quantity of standard solution in our apparatus we have obtained the same result (3·8 cc.) in 10 minutes. (We have also obtained correct results with a micro-apparatus.)

According to this, we are entitled to claim that the apparatus described above, in spite of its technical deficiencies, can be of great use owing to the simplicity of the methods used and the almost automatic character of its work, greatly shortening the time required for many analyses.

There is no doubt that the closed circuit system can be utilised in other ways in work with volatile substances.

VIII. THE DETERMINATION OF SUCROSE BY HANES'S MODIFICATION OF THE HAGEDORN-JENSEN METHOD.

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(*Received December 18th, 1929.*)

Hagedorn and Jensen's micro-method for estimating blood-sugar was modified by Hanes [1929] to make possible the estimation of larger amounts of sugar. Hanes standardised his new procedure for maltose as well as for glucose. It has now been found that the method is also applicable to sucrose.

The first stage in the estimation of sucrose is inversion. A standard solution of invert sugar was prepared by the method of Ling and Rendle [1905]. 0·95 g. of pure sucrose was dissolved in 150 cc. of distilled water; 30 cc. of 0·5 *N* hydrochloric acid were added, and the mixture was heated to boiling point. Boiling was continued for 1 minute. The solution was then rapidly cooled, 30 cc. of 0·5 *N* sodium hydroxide were added and enough distilled water to make 500 cc. This solution was tested by titration with Fehling's solution, using methylene blue as an internal indicator [see Lane and Eynon, 1923]. The concentration of the invert sugar was found to be exactly right, viz. 0·2 g. per 100 cc.

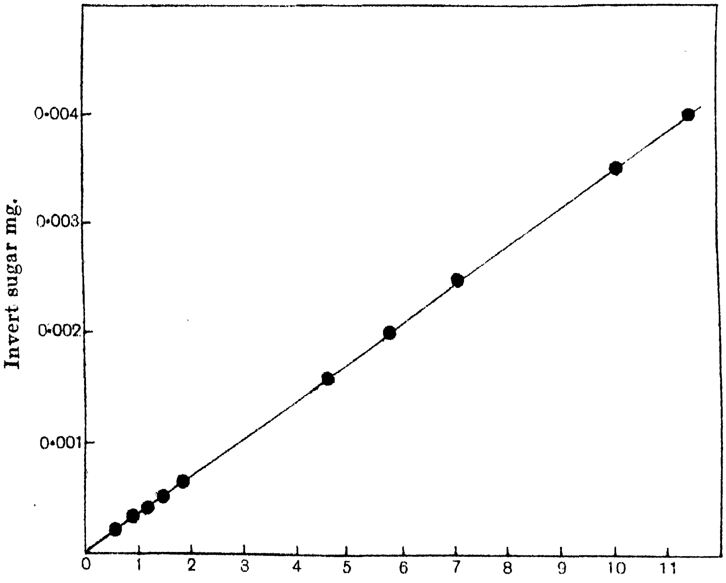

Fig. 1.

This standard solution of invert sugar was used to prepare more dilute solutions of known strength. The thiosulphate equivalent of known volumes of these dilute solutions was determined by the Hanes-Hagedorn-Jensen technique. The estimations were carried out exactly as described by Hanes except that the final volume was in each case made up to 15 cc. instead of 10 cc. Concentrations of invert sugar between 0·2 and 3·5 mg. were used, six estimations being carried out for each concentration. The average of the six readings was used to construct the thiosulphate equivalent curve (Fig. 1). Since it is a straight line, there is a simple relationship between the thiosulphate equivalent and the invert sugar, viz. 1 cc. of 0·01 *N* thiosulphate is equivalent to 0·347 mg. of invert sugar or to 0·329 mg. of sucrose.

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IX. STUDIES ON HEMICELLULOSES.

I. THE HEMICELLULOSES OF WHEAT BRAN.

By FREDERICK WALTER NORRIS and ISAAC ARTHUR PREECE.

From the Department of Biochemistry, University of Birmingham.

(*Received December 20th, 1929.*)

INTRODUCTION.

The term hemicellulose was first proposed by Schulze [1891] as a group name for substances entering into the composition of the cell membrane, which, though in some respects similar to cellulose, yet differ from it in being soluble in dilute alkalis and comparatively easily hydrolysed by dilute mineral acids, yielding pentoses in addition to hexoses.

Although the true physiological significance of the hemicelluloses is for the most part obscure, it would seem that they represent partly structural and partly reserve materials. Thus, reserve hemicelluloses occur in the kernels of seeds, especially such as are poor in their content of starch and fat. In such cases, hexoses predominate among the products of hydrolysis. Hemicelluloses fulfilling probably mainly structural functions occur in seed and fruit husks, wood, straw, etc. The results obtained by earlier workers seemed to indicate that such hemicelluloses were largely composed of pentose residues, xylose and arabinose being the chief hydrolysis products isolated. The recent work of O'Dwyer (see p. 60), however, has shown that the furfuraldehyde-yielding "uronic" acids occur in some, at least, of these hemicelluloses, and it may be that preponderance of pentose residues is not a universal property of substances of this group.

A typical method of attack was that used by Schulze and Tollens [1892], who extracted wheat straw and maize stalks with dilute alkali, precipitating the filtrate with dilute acid. Hydrolysis of the product gave xylose in each case. Schulze [1892] similarly obtained both arabinose and xylose from the brans of wheat and rye. Ulander and Tollens [1906] obtained *d*-mannose and *d*-galactose from certain reindeer mosses which contained no lichenin. Pentosans and methylpentosans were also present. Schulze and Pfenniger [1910], investigating the pods of *Pisum sativum* and *Phaseolus vulgaris*, found them to be rich in hemicelluloses, the amounts of which increased as the pods matured. The products of hydrolysis were galactose and arabinose.

Baker and Pope [1900] obtained mannose and galactose from the hemicellulose of the Indian clearing nut, while the hemicellulose of the ivory nut they showed to be laevulo-mannan. Both these hemicelluloses are to be regarded as reserve substances.

A careful study of the hemicellulose of the wood of American white oak has been carried out by O'Dwyer [1923]. Sawdust from this wood was extracted with 4 % sodium hydroxide, the extract neutralised with glacial acetic acid, and the hemicellulose precipitated by alcohol. The product was purified by precipitation from 2 % sodium hydroxide solution as a copper compound, as described by Baker and Pope [1900], this then being decomposed by treatment with dilute acetic acid. The hemicellulose was shown to contain some 70 % of pentose residues, xylan and araban, the former preponderating. The hexoses, mannose and galactose, were also identified amongst the hydrolysis products.

O'Dwyer [1926], following a method indicated by Clayson, Norris and Schryver [1921], later separated from beech wood two hemicelluloses with distinct physical and chemical properties. Hemicellulose A, obtained by acid precipitation of a sodium hydroxide extract, yielded on hydrolysis xylose and carbon dioxide, evolved under the conditions described by Nanji, Paton and Ling [1925], equivalent to 11 % of glycuronic acid. Hemicellulose B, obtained from the acid filtrate from the above by alcohol precipitation, gave arabinose and carbon dioxide equivalent to 63 % of what was shown to be galacturonic acid.

These results, showing that uronic acids, in some cases at least, enter into the composition of hemicelluloses, and also the possibility that the hemicellulose from a particular source may be split up into a number of fractions, indicate a need for revision of much of the earlier work on these susbtances.

Much confusion in nomenclature exists with reference to these so-called hemicelluloses. This is largely due to the great variety of plant material in which they are to be found and also to the doubt which exists as to their exact function. Schryver recognised the necessity for improvement in the nomenclature of these substances, and recently suggested the term "polyuronide" to describe substances in which uronic acids are found in conjugation with sugars, especially pentoses [Candlin and Schryver, 1928]. The class of "polyuronides" would therefore include pectin and the structural "hemicelluloses" such as those described by O'Dwyer. The older term "hemicellulose" might then be reserved for products which yield no uronic acids on hydrolysis. On this basis of classification, some of the products described in this paper are more correctly described as "polyuronides."

The work herein described aimed at a somewhat detailed investigation of the hemicelluloses of wheat bran. Owing to the ease with which such products as hemicelluloses may become contaminated with other colloidal cell-wall substances, special care is necessary if a pure product is to be obtained. To this end, such substances as pectin and lignin were removed from the tissue, by appropriate extractives, prior to the extraction of the hemicelluloses.

EXPERIMENTAL.

Preparation.

400 g. of the bran are allowed to stand for an hour with 2 litres of water. The mixture is then poured into an equal volume of 1 % ammonium oxalate solution, previously heated on the water-bath to 90°. The temperature is taken back to 90° and is maintained at this level for 2–3 hours. The mixture is allowed to cool, filtered through muslin, and extraction of pectin from the residue is repeated with 0·5 % ammonium oxalate. After filtering, as much liquid as possible is removed at the press.

Removal of lignin is carried out by two extractions with 3 litres of 50 % alcohol containing 1 % of NaOH, each extraction being carried out under reflux for 2 hours. After the second extraction, the filtered tissue is further treated under reflux with 3 litres of neutral alcohol (50 %) for 1 hour. After filtration through muslin, excess liquid is again removed at the press.

The first extraction of hemicellulose is carried out with 3 litres of 4 % NaOH solution. Two or three further extractions with 4 % NaOH should be made from the residue. The combined extracts are filtered carefully, several times, through glass wool.

To the filtered extract is added rather more glacial acetic acid than is necessary to neutralise it. The precipitate obtained at this stage (hemicellulose A) must be separated at the centrifuge. The mother liquor is reserved. The precipitate is repeatedly washed at the centrifuge with water, the early washings being added to the mother liquor. The precipitate is then treated with gradually increasing strengths of alcohol, in the usual way for obtaining a dry product. Drying is completed *in vacuo* over phosphorus pentoxide.

The mother liquor from the above is filtered through paper pulp, after which half a volume of acetone is added to it. This precipitates hemicellulose B, which, after standing, may be filtered off. The filtrate is reserved. The precipitate is redissolved in 4 % NaOH solution, the solution neutralised with glacial acetic acid, and the hemicellulose reprecipitated with half a volume of acetone. After several re-solutions and reprecipitations in this way, the final product is taken up to dryness in the usual manner.

To the filtrate reserved from the above is added a volume of acetone equal to that added previously. The precipitate—hemicellulose C—is purified by dissolving in water and precipitating with an equal volume of acetone, re-solution and reprecipitation being carried out several times and the final product then being taken to dryness.

Yields of the crude hemicelluloses obtained in a typical preparation from 400 g. of bran, calculated on the dry weight of the products, were: hemicellulose A, 8·0 g.; B, 4·5 g.; C, 4·5 g.

Purification.

Hemicellulose A. The crude product is dissolved in 4 % sodium hydroxide solution (1000 cc.), and any undissolved matter is removed at the centrifuge or by filtration through paper pulp. 250 cc. of mixed Fehling's solution are added to the clear liquid, and the copper compound formed is filtered off and decomposed by treatment with dilute hydrochloric acid. Precipitation of the hemicellulose is effected or assisted by the addition of acetone. The product is washed free from acid and copper chloride by three or four changes of acetone, after which drying is completed with alcohol. The product obtained in this way is contaminated with a little starch, but means have not been devised for eliminating this.

Hemicellulose B. Some 5 g. of the crude product are dissolved with constant stirring in boiling water, and the solution is boiled for a further 5 minutes. Any small amount of insoluble residue is filtered off through glass wool and is neglected. The clear solution is cooled, and NaOH is added to give a final concentration of 4 %. 250 cc. of mixed Fehling's solution are added with stirring, and after standing the precipitate—B 1 copper complex—is removed by pouring through muslin. After further filtration through paper pulp, half a volume of acetone is added to the filtrate, and the second precipitate—derived from hemicellulose B2—filtered off. The two precipitates, B1 and B2, are decomposed separately with dilute hydrochloric acid, and acetone is added to effect or assist precipitation of the hemicelluloses. After further washings with acetone, and finally with alcohol, the process of purification described is repeated with each product to ensure complete separation.

Hemicellulose C. This product is dissolved in 4 % NaOH and mixed Fehling's solution added. Although the viscosity of the solution increases greatly, no precipitate is obtained; consequently half a volume of acetone is added, and the precipitate—designated C2 by analogy with B2 above—filtered off, decomposed with hydrochloric acid, and the hemicellulose C2 precipitated, washed, and dried as usual.

General properties of the hemicelluloses.

The four hemicellulose fractions were obtained in the form of white amorphous powders of low ash content (0·3–2·0 %). Hemicellulose A was somewhat soluble in boiling water, while fractions B1, B2 and C2 were almost completely dissolved. The aqueous solution of C2 was laevorotatory. No iodine colorations were obtained, except in the case of fraction A, a suspension of which in water gave a blue colour owing to the presence of a little starch.

Furfuraldehyde estimations were carried out on all fractions, and also decarboxylations by the method of Nanji, Paton, and Ling [1925]. Mean values for a number of determinations are given in the following table, the results quoted being calculated in all cases on the dry, ash-free substance.

Hemicellulose ...	A	B 1	B 2	C 2
	%	%	%	%
Furfuraldehyde	55·0	48·4	3·2	49·0
Carbon dioxide	Nil	1·85	Nil	1·98
Uronic anhydride	,,	7·40	,,	7·92

The crude hemicellulose B yielded 31·1 % of furfuraldehyde. Consequently, fractions B1 and B2 are present approximately in the ratio 2 : 1.

Preliminary hydrolysis.

The yields of hemicelluloses obtained were not sufficient for more than preliminary investigations to be made as to the nature of the sugars liberated on hydrolysis.

The procedure followed in all cases was to boil the hemicellulose under reflux for 4 hours with 1 % sulphuric acid, 50 cc. of the acid being used per g. of hemicellulose. At the end of this time, the hydrolysis liquor was neutralised while still hot with the theoretical volume of a standard strong solution of baryta. The precipitate of barium sulphate formed was filtered off, and the filtrate suitably concentrated *in vacuo.*

Hemicellulose A. The concentrated liquor obtained thus from 5 g. of hemicellulose A was divided into several portions. One portion treated with diphenylhydrazine yielded a white crystalline precipitate of arabinose diphenylhydrazone, melting, after two recrystallisations, at 202°.

From another portion, cadmium xylonobromide was obtained, indicating the presence of xylose.

Repeated attempts to obtain mucic acid, by nitric acid oxidation of some of the sugar syrup, failed, nor was it possible to prepare mannose hydrazone. Likewise, the Pinoff-Selivanoff test for ketohexoses gave a negative result.

An osazone was prepared, which was found to be a mixture. A little of it was insoluble in hot water; examined microscopically this was found to be glucosazone, doubtless derived from the contaminating starch. Examination of the osazone soluble in hot water showed it to be a mixture of xylosazone and arabinosazone, the characteristic form of each being clearly seen.

Thus, it may be supposed that the only sugars obtainable from hemicellulose A on hydrolysis are xylose and arabinose. The detection of these two sugars as the products of hydrolysis of the hemicellulose of wheat bran, which is precipitable by acid, thus confirms the finding of Schulze [1892], whose "wheat bran hemicellulose" corresponds to this fraction.

*Hemicellulose B*1. The chief product of hydrolysis of this substance would appear to be xylose. A large amount of cadmium xylonobromide was obtained on treating a portion of the hydrolysis liquor according to Bertrand's method. Another portion of hydrolysis liquor gave a very small amount of arabinose diphenylhydrazone, melting after recrystallisation at 202–203°.

An osazone was prepared, and a little of it, too small in amount for further examination at this point, was found to be insoluble in hot water. The residue was practically pure pentosazone, melting after several recrystallisations at 155°.

In an endeavour to ascertain the nature of the insoluble osazone, the hydrolysis liquid from a further 5 g. of B1 was evaporated *in vacuo*, just to dryness, and the sugar extracted twice with 50 cc. portions of absolute alcohol. The residue was then extracted with 80 % alcohol. The combined absolute alcohol extracts and the 80 % extract were separately evaporated, and the sugars taken to dryness by repeated treatment with absolute alcohol, drying being completed *in vacuo*. The bulk of the sugar was extracted by the absolute alcohol, and the dry product from this gave $[\alpha]_{D}^{20}$ - 26° for $c = 1 \cdot 6$.

The osazone obtained from this fraction was a mixture. The portion insoluble in hot water was repeatedly extracted with hot water to remove pentosazone. It was recrystallised from 50 % alcohol, and under the microscope was seen to crystallise in clusters of thick yellow needles, quite distinct in appearance from the osazones of glucose and galactose. The melting point of the purified osazone was 158°. The pentosazone, doubtless xylosazone, melted after recrystallisation at 155°.

It seems not improbable that the insoluble "osazone" here obtained was derived from glycuronic acid. Mayer [1900] found that glycuronic acid treated with excess of phenylhydrazine yielded a compound melting at 159-164°. Again, Giemsa [1900] obtained a glycuronic acid phenylhydrazine derivative melting at 160°, crystallising in yellowish needles, insoluble in water, and recrystallisable from dilute alcohol Galacturonic acid, on the other hand, is said to yield no crystalline derivatives with phenylhydrazine [see Ehrlich, 1917].

The sugar soluble in 80 % alcohol gave two similar osazone fractions.

Other sugar tests than those described having yielded negative results, it appears that the products of hydrolysis of hemicellulose B1 consist chiefly of xylose, accompanied by a little arabinose and also a uronic acid, possibly glycuronic acid, though the evidence for this is at present incomplete. At the same time, the furfuraldehyde yield from the hemicellulose, viz. 48·4 %, is too low to be accounted for in this way, and it is possible that another sugar present has escaped detection.

Hemicellulose B2. This yielded but 3·2 % of furfuraldehyde, while uronic acids were shown to be absent. Consequently, on hydrolysis, a small amount of pentose should be obtained, the residue of the hydrolysis product being hexose. Hydrolysis was carried out in the usual way on some 2 g. of B2.

From a portion of the neutral hydrolysis liquor an osazone was prepared, the bulk of which was insoluble in hot water. This osazone was found to crystallise in sheaves of needles, resembling glucosazone. After recrystallising from 50 % alcohol it gave a melting point of 205°. Glucosazone melts at this temperature. The small amount of pentosazone formed, soluble in hot water, was not examined.

Attempts to obtain mannose hydrazone from another portion of the hydrolysis liquor failed, while the Pinoff-Selivanoff test for ketoses likewise gave a negative result.

It seems, therefore, that hemicellulose B2 is a glucosan, admixed with a little pentosan. The amount of the hemicellulose available, however, was not sufficient to allow of further test.

Hemicellulose C2. A copious white crystalline precipitate was obtained when a little of the hydrolysis liquor from hemicellulose C2 was treated in the usual way for the production of arabinose diphenylhydrazone. After two recrystallisations from 60 % alcohol this product melted at 203°, establishing the presence of arabinose.

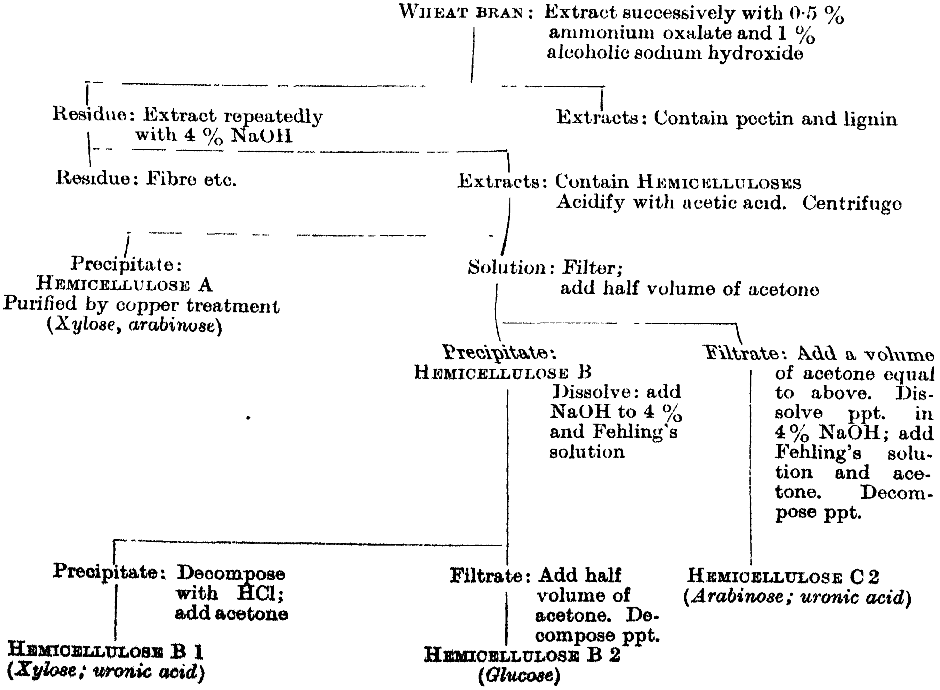
A very small amount of cadmium xylonobromide was obtained from another portion of the hydrolysis liquor. Mucic acid could not be obtained.

An osazone was prepared, and proved to be a mixture. A portion insoluble in hot water, after purification and recrystallisation from 50 % alcohol, was found to have a crystalline form resembling that of the insoluble phenylhydrazine derivative described above as obtained from the products of hydrolysis of hemicellulose B1. It melted at 161–163°. Hence this too may be a derivative of glycuronic acid. The osazone soluble in hot water formed along with this was arabinosazone, contaminated with a little xylosazone, and melting at 155°.

Thus, wheat bran hemicellulose C2 is an araban, associated with some 8 % of uronic acid anhydride, possibly glycuronic anhydride, and contaminated with a little xylan.

DISCUSSION.

The following scheme indicates diagrammatically the method of preparation of the hemicelluloses, the products of hydrolysis being shown in brackets.


WHEAT BRAN: Extract successively with 0·5 % ammonium oxalate and 1 % alcoholic sodium hydroxide
Residue: Extract repeatedly with 4 % NaOH
Extracts: Contain pectin and lignin
Residue: Fibre etc.
Extracts: Contain HEMICELLULOSES Acidify with acetic acid. Centrifuge
Precipitate: HEMICELLULOSE A Purified by copper treatment (*Xylose, arabinose*)
Solution: *Filter;* add half volume of acetone
Precipitate: HEMICELLULOSE B Dissolve: add NaOH to 4 % and Fehling's solution
Filtrate: Add a volume of acetone equal to above. Dissolve ppt. in 4% NaOH; add Fehling's solution and acetone. Decompose ppt.
Precipitate: Decompose with HCl; add acetone
Filtrate: Add half volume of acetone. Decompose ppt.
HEMICELLULOSE C2 (*Arabinose; uronic acid*)
HEMICELLULOSE B1 (*Xylose; uronic acid*)
HEMICELLULOSE B2 (*Glucose*)

Four hemicellulose fractions with widely differing characteristics have thus been separated from wheat bran, two of them containing small amounts of uronic acid residues.

Hemicellulose B2 is of particular interest, both from its mode of separation, and from the fact that it is practically a pure hexosan. The preliminary hydrolysis described would appear to suggest that it is a glucosan. Though glucosan is not usually to be regarded as a constituent of hemicelluloses (except the amylohemicelluloses, rather different types of substance), various workers have claimed to obtain glucose from hemicellulose hydrolysis products. Thus, Schulze and Castoro [1903] obtained from certain hemicelluloses a sugar convertible to glucosazone which they regarded as probably glucose. Also, glucose has been stated to arise, along with mannose and methylpentose, when the hemicellulose-like yeast gum is hydrolysed [see Hashitani, 1927].

The results in the case of hemicellulose B1 must be considered unsatisfactory, since it would appear that a hexose has escaped detection. Xylose, arabinose, and a uronic acid were shown to be present, but the figures obtained for furfuraldehyde and carbon dioxide are so low that they indicate the presence of another constituent. The very small amounts of the individual fractions available renders the isolation of hydrolysis products a difficult problem.

On the other hand, furfuraldehyde and carbon dioxide numbers are almost completely accounted for on the assumption that this substance is a urono-araban containing some 8 % of uronic anhydride.

SUMMARY.

A general method of preparation and purification of hemicelluloses is described.

The hemicelluloses of wheat bran yield four fractions, chemically and physically distinct, two of them containing small percentages of uronic acid residues.

Preliminary hydrolyses of the four products have been carried out. Hemicellulose B2 appears to be a glucosan, whilst the other fractions are largely composed of pentose residues.

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X. THE SOLUBILITY OF GLYCOGEN.

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In the course of an investigation on glycogen, the solubility of a sample prepared in the usual way by boiling tissue with 60 % KOH was compared with that of a sample prepared from an aqueous extract of tissue dehydrated by alcohol, and it was observed that the former sample reached a stable value very much more rapidly than the latter. It was therefore decided to prepare glycogen from several sources, without boiling with potassium hydroxide and to determine the solubilities of the products obtained. In the course of the work it was found necessary to revise the method employed for estimating glycogen, and, in order to do this, the solubility of glycogen in aqueous alcohol was investigated.

Method of preparation.

Since the usual method of preparing glycogen by boiling tissue with 60 % potassium hydroxide appeared to affect the solubility of the product, it was considered advisable to use a method less likely to have a chemical action on the carbohydrate complex. Dehydration of the tissue by alcohol and subsequent extraction with water was the method selected, except in one case (frog muscle) where the tissue was extracted with 4 % trichloroacetic acid, and the glycogen precipitated with alcohol. Three sources of glycogen were used: mussel (whole body), frog (skeletal muscle) and rabbit (liver). The method employed varied slightly in each case.

Mussel (Mytilus edulis). Mussels were killed, and the tissue was dehydrated, by being put into alcohol. For the first batch boiling 80 % alcohol was used, for the second, cold 80 % alcohol. The solid residue was filtered off, dried with absolute alcohol and with ether, exposed to the air to remove all traces of ether, and finally ground in a mortar. Glycogen was extracted from this powder with water: the proteins, denatured by the treatment with alcohol, were soluble only to a small extent.

To test the completeness of extraction, a portion of the mussel powder was extracted 18 times with small quantities of water at 0°, and the glycogen in each extract and in the residue estimated separately (Fig. 1, curve *a*). The experiment was repeated, using water at 25° (curve *b*), but the amounts extracted were not appreciably greater. Grinding with sand between each extraction gave much more satisfactory results (curve *c*), and it was concluded that the difficulty of extraction was mechanical. From Fig. 1 it is apparent that the seventh and subsequent extracts contain negligible amounts of glycogen; in later work, therefore, the mussel powder was extracted six times.

Glycogen values are given throughout this paper in terms of the reducing sugar found after 3 hours' hydrolysis by 2·2 % by wt. hydrochloric acid. It is usual to multiply this by 0·927 to convert to glycogen. This assumes a 97 % hydrolysis, but working with a purified sample of glycogen prepared by Dr Slater, it was never found possible to reach so high a value, the mean obtained being 94 %, corresponding to a conversion factor 0·957.

The glycogen in the water extract was purified by precipitation with two volumes of alcohol.

The nitrogen and phosphorus contents of successive precipitates were examined, and it was found that the ratio of the nitrogen and phosphorus to glycogen fell considerably after the first precipitation, showed no decided change after the second, and after the third precipitation the nitrogen values rose, phosphorus remaining nearly steady (Table I). This unexpected result was repeated in each preparation examined, and suggests that the glycogen molecule in the natural state may contain nitrogen, and possibly also phosphorus. For the third precipitation it was necessary to add an electrolyte to coagulate the glycogen; a few drops of a normal solution of potassium acetate were used.

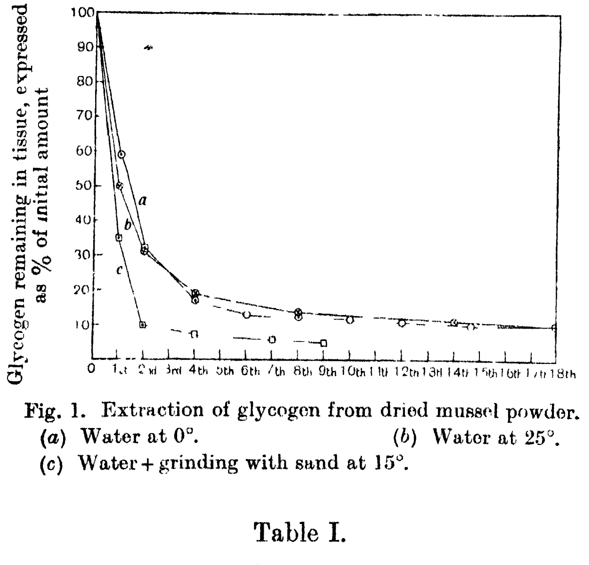

Fig. 1. Extraction of glycogen from dried mussel powder.
(*a*) Water at 0°. (*b*) Water at 25°.
(*c*) Water + grinding with sand at 15°.

Table I.

Ratio mg. phosphorus and mg. nitrogen to 100 mg. glycogen.

	Untreated extract		After 1 ppt.		After 2 ppt.		After 3 ppt.	
	P	N	P	N	P	N	P	N
Prep. A	0·91	1·91	0·20	0·55	0·21	0·56	0·17	0·98
„ B	0·85	1·74	0·75	0·57	0·63	0·54	0·64	0·70

In order to remove small amounts of protein that might still be associated with it, the glycogen was dissolved in 4 % trichloroacetic acid, filtered and dialysed.

After three precipitations with alcohol, dialysis of the product produced a rise in the ratio of nitrogen to glycogen. If the glycogen were first dissolved in 4 % trichloroacetic acid, the small amount of insoluble matter filtered off, and the solution dialysed, there was a larger rise. If, instead of being dissolved in trichloroacetic acid, the glycogen were boiled for 2 hours with 60 % potash, the ratio of nitrogen to glycogen fell very considerably (Table II).

Table II.

Ratio mg. nitrogen to 100 mg. glycogen.

Prep. C after 2 ppt. with 66 % alcohol	After dialysis	After dissolving in 4 % trichloroacetic acid with subsequent dialysis	After boiling with 60 % potash
0·77	0·81	0·83	0·30

After dialysis the glycogen was precipitated a third time in 66 % alcohol and then dried in a vacuum desiccator over calcium chloride. This should give the half-hydrate, $C_6H_{10}O_5$, $\frac{1}{2}H_2O$ [Slater, 1924].

Rabbit. The rabbit liver was removed immediately the animal was killed and was minced into ice-cold 94 % alcohol, filtered and the residue re-extracted with ice-cold alcohol, filtered and the residue dried in air and ground in a mortar. Glycogen was extracted from this powder in the same manner as for mussels.

Frog. The frogs' muscles were ground in 4 % trichloroacetic acid and the extract treated as the water extract from mussel powder, except that the glycogen was not again dissolved in trichloroacetic acid.

Method of determining solubility.

Preliminary experiments showed that glycogen did not go into solution completely for several days. This made it necessary to ensure that the material should remain sterile during the time of the experiment. Small two-necked flasks were used, one neck closed with a rubber cap, the other stoppered with cotton wool and covered with a glass cap. A small amount of glycogen (1 to 0·3 g.) was placed in the flask and sterilised for 30 minutes in steam on 3 consecutive days; an amount of sterile water, insufficient to dissolve all this glycogen, was injected through the rubber cap, and the flask was placed in a thermostat. At intervals of 6 to 60 hours small amounts (about 0·5 cc.), were withdrawn by means of a hypodermic syringe, placed in a centrifuge cup, covered with a drop of liquid paraffin to prevent evaporation, and centrifuged for 20 minutes. 0·1 cc. of the solution was used for the estimation of the glycogen. That the solutions remained sterile was shown by plating out a drop on agar at the conclusion of the experiment. (Previous experiments with unsaturated glycogen solutions showed that the strength of the solution was not altered by centrifuging in these conditions.)

Method of estimating glycogen.

Since the quantities of glycogen available were small it was necessary to use a micro-method for estimation. The glycogen was still associated with appreciable quantities of nitrogen and phosphorus, and was therefore possibly contaminated by reducing substances, so it was considered desirable to use the full method of estimation by boiling with 60 % potassium hydroxide and not merely to hydrolyse the solution with acid.

With the exception of the method of de Jongh and Planelles [1924], depending on the turbidity of glycogen solutions precipitated by alcohol in the presence of ether, which is not suitable for accurate estimations, all micro-methods for the estimation of glycogen are modifications of Pflüger's method [1904], and depend on the destruction of lower carbohydrates by boiling with 60 % potassium hydroxide, and precipitation of glycogen from the resulting solution with alcohol. Evans [1925] has pointed out that when estimating small quantities of glycogen in a considerable mass of tissue, involving the use of relatively large volumes of solution, there is a loss of glycogen, increasing with increasing volume of fluid, pointing strongly to the solubility of glycogen in aqueous alcohol, at least in the presence of a high concentration of potash. Holmes and Holmes [1926] carried out some experiments to test the recovery of glycogen added to a potassium hydroxide-alcohol mixture of the concentration used in their estimations, and obtained on the average a recovery of over 100 %, and concluded that there is no appreciable loss through solubility of glycogen in alcohol. Possibly they obtained such good values because they added solid glycogen, instead of precipitating from a solution, and, as the results of the solubility determinations show, glycogen may be very slow in dissolving. In a modification of the Pflüger method introduced by Bierry and Gouzon [1928], glycogen is not precipitated after alkaline hydrolysis, but acid is added and an acid hydrolysis carried out on the same solution. Proteins are afterwards removed with mercuric nitrate, and the sugar is estimated. This procedure avoids loss of glycogen by solution in alcohol, but might cause an additive error, since any reducing substance not destroyed in either the alkaline or the acid hydrolysis, and not precipitated by mercuric nitrate, would be estimated as sugar.

To find whether any glycogen remained in solution after precipitation with alcohol, when using a micro-modification of the Pflüger method, the residual alcoholic solution from a glycogen estimation (the tissue used in this, and in all other estimations when working out the method for estimating glycogen, was, for convenience, skeletal muscle from the frog) was evaporated nearly to dryness, washed with 1 cc. of absolute alcohol and centrifuged, and the glycogen estimated in the residue by acid hydrolysis and determination of the reducing power.

Table III.

Apparent solubility of glycogen from frog muscle in 75 % alcohol.

0·2–0·3 g. muscle (or in last 3 experiments 0·1 cc. glycogen solution), were boiled with 2 cc. 60 % potassium hydroxide for 2½ hours, neutralised with glacial acetic acid, filtered and 3 volumes absolute alcohol added, making 20 cc. in all.

mg. glycogen ppt. by alcohol	mg. glycogen recovered from alcohol	Apparent solubility of glycogen in 75 % alcohol mg. per 100 cc.
0·59	0·166	0·83
0·88	0·128	0·64
0·59	0·166	0·83
1·10	0·227	1·14
1·09	0·189	0·95
0·065	0·091	0·46
0·054	0·295	1·48
—	0·241	1·21
0·159	0·172	0·86
9·50	0·143	0·72
9·50	0·156	0·78
6·83	0·197	0·99

The results (Table III), show clearly the presence of some reducing substance, probably, at least in part, glycogen. A reducing substance stable to alkaline hydrolysis, soluble in 75 % alcohol, and relatively insoluble in absolute alcohol would be included in this estimation. If there should be such a substance present in the muscle in variable quantity, it would account for the irregularity of the results.

Methyl alcohol and acetone were also tried as precipitants for glycogen, but failed to give as good results as ethyl alcohol.

As these results indicated that glycogen had an appreciable solubility in 75 % alcohol, but did not give accurate figures, it was decided to study the problem of the solubility of glycogen in aqueous alcohol using purified glycogen and varying strengths of alcohol, so that the influence of other substances present in muscle should be eliminated.

Solubility of glycogen in aqueous alcohol.

In practice it was found best to estimate the solubility by an indirect method. A known quantity of glycogen in aqueous solution was treated with alcohol in amounts calculated to give 50, 60, 66, 75, 80 and 90 % alcohol by volume. The glycogen precipitated by this treatment was separated by centrifuging and estimated. The solubility of glycogen in the aqueous alcohol was calculated from the difference between the amount of glycogen precipitated and the amount originally taken. On account of the slight solubility of glycogen, very small quantities must be used in order to show a difference that can be measured with any degree of accuracy. The glycogen used was a highly purified sample from *Mytilus*, kindly supplied by Dr Slater. It is generally agreed that to precipitate pure glycogen satisfactorily from aqueous solution by the addition of alcohol is difficult. The turbid solution will not coagulate and settle with any regularity, even after centrifugalisation for 30 minutes. One drop of a 60 % potassium chloride solution was therefore added, making a final concentration of 0·6 % potassium chloride (Fig. 2, curve *a*).

When estimating glycogen after boiling with potassium hydroxide there is always a high concentration of salt or of alkali present, so the solubility was also determined in the presence of 21 % potassium acetate, 42 % potassium trichloroacetate, 15 % potassium chloride, and 15 % potassium hydroxide, these being approximately the concentrations reached when estimating glycogen. In each case the electrolyte was added to the glycogen solution before the alcohol, and subsequent procedure was that described above. The results are shown in Fig. 2. The solubility in the presence of 4 % trichloroacetic acid was also determined, since this concentration of trichloroacetic acid is frequently used for extracting tissue.

In previous methods of estimating glycogen the concentration of alcohol used for precipitating glycogen has varied considerably. Pflüger [1904] used 57 %; Kahn [1922] in a micro-method 52·5 %; Evans [1926] for plain muscle 66 %. From the curves in Fig. 2 it is clear that it is useless to employ higher concentrations of alcohol than 66 % when the original volume of solution is fixed, since the loss due to increased final volume more than counterbalances the gain from decreased solubility. When the final volume is the limiting factor it is definitely better to use 66 % rather than 75 %, as it is possible to use a larger initial volume and the percentage loss is lessened.

Pflüger [1904] precipitated glycogen from the alkaline solution after diluting with two volumes of water; in some more recent methods the alkali is neutralised, usually with glacial acetic acid, in order to precipitate protein, and this is filtered off before alcohol is added. It is apparent from Fig. 2 that glacial acetic acid is not a suitable acid to use, as the solubility of glycogen is higher when potassium acetate is present than in the presence of either potassium trichloroacetate, potassium chloride or un-neutralised KOH. It would seem that there is little to choose from the point of view of solubility alone between these last three conditions. It is probably better to neutralise, as this undoubtedly removes considerable quantities of protein. When hydrochloric acid is used, although the solubility of glycogen is least, a further complication is introduced as the potassium chloride formed is only slightly soluble in alcohol, and large quantities of salt are precipitated along with the glycogen. This did not interfere with the acid hydrolysis, or with the estimation of sugar by the Hagedorn and Jensen method, but might with other methods. Since trichloroacetic acid is a definite protein precipitant, and its potassium salt is soluble in alcohol, it was decided to use this acid. Table IV shows a comparison of the result of neutralising with each of the three acids in an estimation of glycogen in muscle.

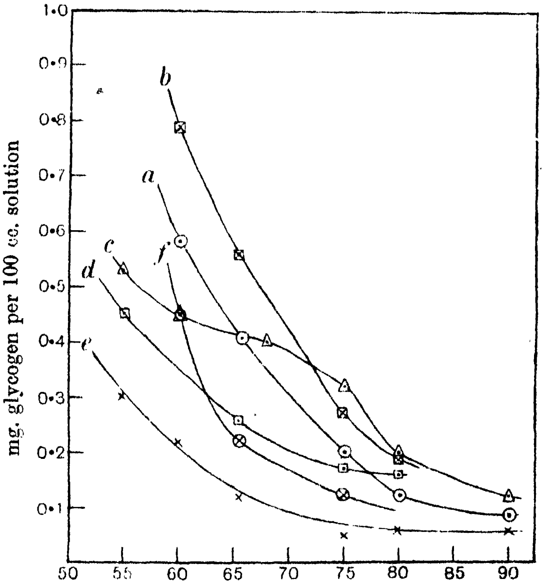

Fig. 2. The solubility of glycogen in aqueous alcohol.

(a) Alcohol + 0·6 % KCl.
(b) Alcohol + 4 % CCl_3COOH.
(c) Alcohol + 21 % CH_3COOK.
(d) Alcohol + 42 % CCl_3COOK.
(e) Alcohol + 15 % KCl.
(f) Alcohol + 15 % KOH.

Table IV.

After boiling frog muscle with potassium hydroxide the solution was diluted, three equal parts taken and neutralised with hydrochloric, glacial acetic and trichloroacetic acids respectively, the precipitates (of protein) were removed by centrifuging and the glycogen in an aliquot part of each solution precipitated with two volumes of alcohol and estimated.

	mg. glycogen found when alkaline digest is neutralised with				
Final vol. of solution cc.	Glacial acetic (*a*)	Hydrochloric (*b*)	Trichloroacetic (*c*)	*b* − *a*	*c* − *a*
6	0·867	1·283	1·231	0·416	0·364
6	0·495	0·640	0·629	0·145	0·134
6	0·808	0·938	0·996	0·130	0·188
6	0·496	0·472	0·976	−0·024	0·480
9	9·06	8·87	—	−0·19	—
9	3·80	4·21	—	0·41	—
9	4·74	—	4·92	—	0·18
9	8·71	—	8·75	—	0·04

The final procedure adopted was as follows. The solution was boiled with 60 % potassium hydroxide for 2½ hours, cooled, neutralised with 40 % trichloroacetic acid, the final adjustment being made with dilute potassium hydroxide, so that the solution was faintly violet to bromocresol purple, the solution was made up to a known volume, centrifuged, and two volumes of alcohol were added to an aliquot part. Bromocresol purple was chosen as an indicator since it changes colour almost at neutrality, and since it is possible to see the colour even when working on muscle, which gives a brown solution. The glycogen precipitate was separated by centrifuging, washed once with absolute alcohol, hydrolysed with 2·2 % hydrochloric acid, and the sugar estimated either by the original Hagedorn and Jensen method [1923], or by Hanes's [1929] modification for use with larger quantities. It is advisable to wash the glycogen with absolute alcohol, since even 80 % dissolves an appreciable fraction. A correction can be added for the glycogen lost by solution in the alcohol when precipitating, it can be found by reference to the potassium trichloroacetic acid curve (Fig. 2, curve *d*). Table V shows the result of estimating a solution of purified glycogen, (*a*) by acid hydrolysis, (*b*) by the method described above. The recovery of glycogen added to an estimation on frog muscle is also shown. The glycogen solution was added to a portion of the alkaline digest before neutralisation. In each case the recovery is low: this may be due to some substance present in muscle, which affects the solubility of glycogen, as noted above.

Table V.

mg. glycogen found in 2 cc. solution.		
Estimated by acid hydrolysis	Estimated as described in text	Added in an estimation on muscle
1·36	1·30	1·31
1·34	1·40	1·28
2·12	2·14	2·02
2·14	2·12	2·26
2·17	2·05	—
2·14	2·04	—

Solubility of glycogen in water.

First the solubility of mussel glycogen, prepared as described, *i.e.* without boiling with alkali, was compared, at 20°, with that of a similar sample boiled for 2½ hours with 60 % potassium hydroxide. The results are shown in Fig. 3 (curves *a* and *b*). When the experiment was repeated, using a second preparation of glycogen from the same batch of mussel powder, the curves produced by plotting the results were not identical with those of the first preparation, but of a similar type (Fig. 3, curves *c* and *d*). Glycogen prepared from a second batch of mussel powder, prepared similarly to the first, except that the mussels were killed and dehydrated by cold alcohol instead of boiling alcohol, gave a curve intermediate between those of the first two preparations (Fig. 3, curve *e*). In each case the solubility of glycogen which had been boiled with alkali had reached a practically stable value at the first estimation, whereas the glycogen not boiled with alkali required from 3 to 4 days for maximum solubility to be reached. In the first experiment after 5 days at 20° the solutions were heated to 50°, and cooled again. The solubility of both samples of glycogen returned to the same value.

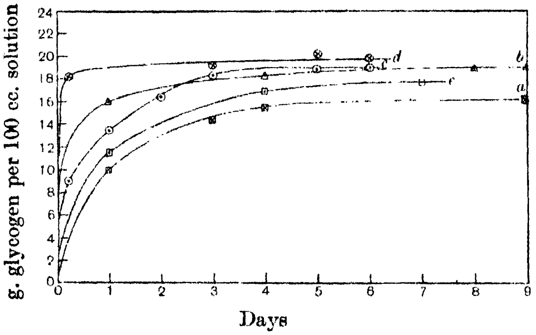

Fig. 3. Solubility of glycogen in water at 20°.

Prep. I. (*a*) Not boiled with KOH 0·92 mg. nitrogen per 100 mg. glycogen.
(*b*) Boiled with KOH 0·18 ,, ,, ,, ,,
Prep. II. (*c*) Not boiled with KOH 0·61 ,, ,, ,, ,,
(*d*) Boiled with KOH 0·06 ,, ,, ,, ,,
Prep. III. (*e*) Not boiled with KOH 0·50 ,, ,, ,, ,,

The solubility of mussel glycogen was then determined at 0° and 37° (Table VI). At 37° all the glycogen (0·4 g.) had dissolved in the water added (1 cc.) forming a jelly. A portion of this jelly contained 40 % glycogen, so probably the glycogen was evenly distributed and is soluble at least to this extent at 37°.

Table VI.

Solubility of glycogen in water after 7 days at

0°	20°	37°
16 %	17·7 %	>40 %

The solubility of glycogen from mussels, frog muscle and rabbit liver was compared at 20° (Fig. 4). The sample from rabbit liver had a higher solubility than either of the samples from cold blooded animals.

It seems possible that the increasing solubility of the samples not boiled with potassium hydroxide, reaching a value that approximates, but does not quite reach, that of the samples boiled with alkali, might be due to a depolymerising influence of water. If this is so, the boiling with alkali exerts the same effect at a much greater rate, and possibly to a greater extent. Schmid, Ludwig and Pietsch [1928] observed a somewhat similar effect when determining the molecular weight of glycogen in liquid ammonia, as their glycogen did not reach a steady state for half an hour, starting from a very large molecular weight and reaching a value of 180. Their samples had presumably all been boiled with potassium hydroxide, since two were prepared commercially and no special mention is made of the preparation of the third sample (from liver). Possibly if estimations of the solubility of the glycogen boiled with potassium hydroxide had been made during the first half hour a rising value would have been obtained, but there would have been no certainty that this was not due to the normally slow solution of a colloid. This does not necessarily suggest that glycogen in its natural state is polymerised to a large extent, since the glycogen prepared without boiling with alkali had been treated with alcohol in order to purify it, and it might well be this treatment which caused a high degree of polymerisation, if such existed. It is also possible that the nitrogen content of the glycogen is connected with the differences in solubility, as in most cases the rate of solution and the solubility vary inversely with the nitrogen content.

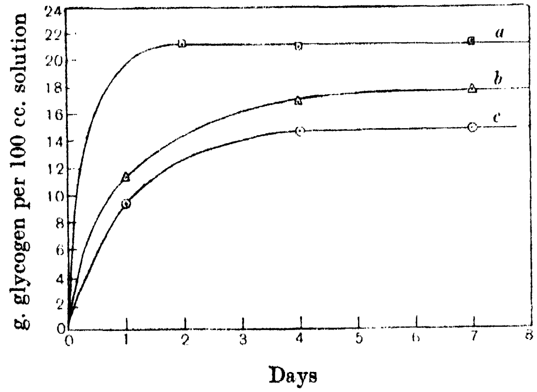

Fig. 4. Solubility of glycogen in water at 20°.
(a) Glycogen from rabbit; 0·12 mg. nitrogen per 100 mg. glycogen.
(b) „ „ mussels; 0·50 mg. „ „ „
(c) „ „ frog; 1·13 mg. „ „ „

SUMMARY.

1. Glycogen from mussels, prepared without boiling with alkali, has been found to take from 3 to 4 days to reach saturation in water, whereas a similar sample boiled for 2½ hours with 60 % potassium hydroxide, reaches a slightly higher value within a few hours.

2. The solubility in water of mussel glycogen, not boiled with potassium hydroxide, at 0° is 16 %, at 20° 17·7 % and at 37° at least 40 %.

3. Glycogen from skeletal muscle of frogs has a solubility in water of 14·9 % at 20°, that from rabbit liver 21 % at 20°.

4. Glycogen is slightly soluble in aqueous alcohol, the concentration and nature of any electrolyte present having an influence on the value. Curves have been obtained showing the decreasing solubility of glycogen with increasing concentration of alcohol in the presence of potassium acetate, potassium trichloroacetate, potassium chloride, potassium hydroxide and trichloroacetic acid.

5. A micro-modification of Pflüger's method of estimating glycogen, based on the results of the experiments on the solubility of glycogen in aqueous alcohol, and employing a correction for this solubility, is described.

6. When glycogen prepared from mussels is precipitated with alcohol, after two precipitations the nitrogen content of the preparation increases, the phosphorus content remaining nearly constant.

I wish to express my thanks to Professor J. C. Drummond for his interest and advice in this work. The cost of the research has in part been defrayed by a grant from the Government Grant Committee of the Royal Society.

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XI. FURTHER EVIDENCE FOR A THIRD ACCESSORY "B" FACTOR.

By VERA READER.

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(*Received December 30th, 1929.*)

In a recent publication [Reader, 1929] evidence was presented for the division of the vitamin-B complex into three components all necessary for the nutrition of the rat. It was shown that two of these were destroyed by autoclaving yeast extract at p_H 9 for one hour at 120°. Further evidence has now been obtained for the separate existence of these three factors, since it has been possible to concentrate two of them, and to find that the third must still be added to enable the animals to grow to maximum adult size.

Throughout this paper the following nomenclature is used.

Vitamin B_1 is the antineuritic vitamin, concentrated by the process of Kinnersley and Peters [1927] to the stage involving its extraction from charcoal by $N/10$ HCl.

Vitamin B_2 is the anti-pellagra vitamin of Goldberger *et al.* [1926] as interpreted by Hassan and Drummond [1927]. It is considered to be the factor present in alkaline autoclaved yeast extract. The term "anti-dermatitis" is probably preferable for vitamin B_2 since some evidence in this laboratory suggests that "pellagra" is due to a combined deficiency of vitamins $B_2 + B_3$.

Vitamin B_3 is the second alkali-labile factor, obtained from the mercuric sulphate precipitate in the Kinnersley and Peters process for vitamin B_1. Williams and Eddy [1928] have styled this third factor for the pigeon vitamin B_3; I have adhered to the nomenclature of my original paper, pending a consideration of the question by the Biochemical Societies concerned.

EXPERIMENTAL.

The preparation of animals was exactly as described in the previous paper [Reader, 1929]. The basal diets were also similar. The vitamin B_1 used was kindly supplied by Mr Kinnersley. Unless otherwise stated 3-pigeon-day-doses (approx. 1·0 mg.) were given to each rat by the mouth daily. The concentrates used had been shown to be free from vitamin B_3 [Reader, 1929].

The vitamin B_2 was supplied as yeast extract which had been heated at 120° for one hour at p_H 9. After being carefully neutralised it was mixed with the diet. The equivalent of 6 % of the original extract was found to be sufficient and was regarded as a unit value. In certain experiments (see Fig. 1 *a*), when the animals had come to constant weight on diets containing vitamins $B_1 + B_2$, the daily allowance of each of these components was doubled over a weekly period to make certain that the cessation of growth was due to the absence of a third factor and not to an insufficient quantity of either of the two already being supplied.

In the search for vitamin B_3, the final filtrate from the Kinnersley and Peters process for vitamin B_1 was first tested for vitamin B_3 activity. As it gave a negative result, all the various discarded precipitates obtained during the process were freed from metals and tested. Only the extract from the mercuric sulphate precipitate gave a positive test. It has since been found possible to recover 75 % of the vitamin B_3 present in the original extract. In the experiments cited in this paper the total organic solids given daily as vitamin B_3 varied from 1 to 2 mg. This quantity was found to contain less than 1/15 of a daily dose of vitamin B_1. Further details of the concentration and purification of vitamin B_3 will be published shortly.

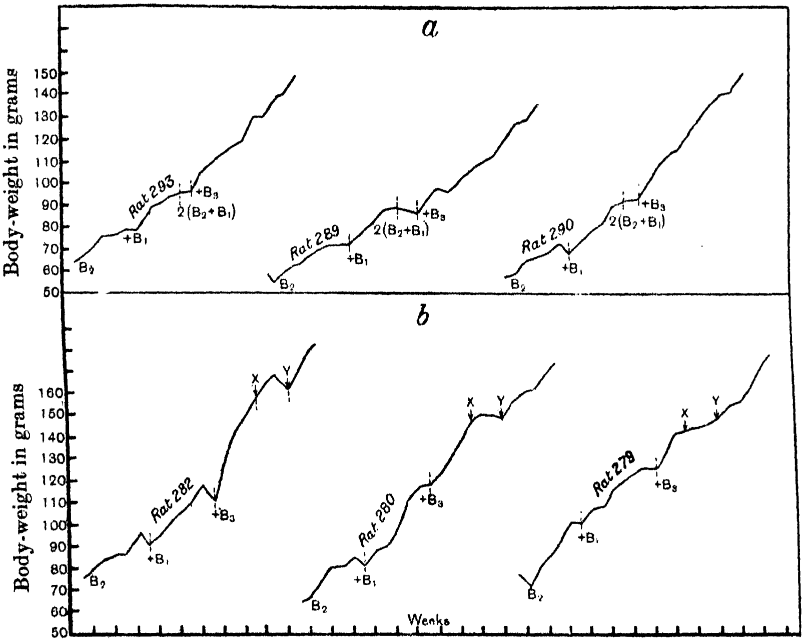

Fig. 1 *a* and *b*. Showing that vitamin B_3 is necessary to supplement vitamins $B_1 + B_2$. At *X*, Fig. 1 *b*, supply of vitamin B_3 was cut off, but resumed again at *Y*.

Ninety rats were used for this work. Two types of experiments were carried out.

Group A, Figs. 1*a* and 1*b*. Rats were brought to constant weight on the basal diet + vitamin B_2: growth usually ceased at the end of 2–3 weeks. Then vitamin B_1 was given daily. An immediate temporary response occurred, the rats continuing to grow for another 2 or 3 weeks. Then no further response could be obtained by doubling the supply of vitamin B_1 or of vitamin B_2 or of both. The animals were then ready for testing one of the various vitamin B_3 preparations: the extract was administered daily by the mouth. The results in Figs. 1*a* and 1*b* were obtained with a vitamin B_3 preparation extracted from the mercury precipitate; from which it must be concluded that some factor necessary for the nutrition of the rat is present in this precipitate. At point *X*, Fig. 1*b*, the supply of vitamin B_3 was cut off, but resumed again at *Y*.

Group B, Fig. 2. The second set of experiments was designed to show that some essential component still remained in the yeast extract after heating at 120° for one hour at p_H 9 and that normal growth could be obtained without adding excess of the yeast extract so treated. The rats were brought to constant weight on the basal diet alone, *i.e.* without any "B" vitamins. Then vitamins B_1 and B_3 were both given daily in quantities which had proved to be sufficient in the previous type of experiment. Practically no growth occurred. At point *K*, Fig. 2, the supplies of vitamins B_1 and B_3 were doubled. The weight was still not increased. When the unit quantity of vitamin B_2 was added, normal growth was immediately resumed.

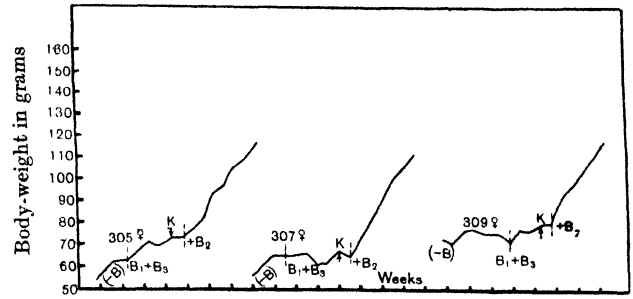

Fig. 2. Showing that vitamin B_2 is necessary as supplement for vitamins $B_1 + B_3$. At point *K* vitamins $(B_1 + B_3)$ were doubled.

DISCUSSION.

In the earlier paper [Reader, 1929] three accessory "B" factors were postulated upon the evidence that addition of vitamin B_1 concentrates to an alkaline autoclaved yeast extract did not induce normal growth, and that growth was not improved by increasing either the vitamin B_1 concentrate or the autoclaved extract. It still remained to be shown that the hypothetical vitamin B_3 could be separated from the other two components. This has now been done.

Concentration of vitamin B_3 has been made possible because it appears in the mercuric sulphate precipitate, suggesting that the factor itself forms an insoluble mercury salt, or a double salt with mercuric sulphate. The curves of Fig. 1 demonstrate that the factor in question is present in the mercury precipitate and those in Fig. 2 that, in addition to this, another factor is required to supplement the antineuritic vitamin; this factor is stable to alkaline hydrolysis for one hour at 120° and is here called B_2. It has been stated by Williams, Waterman and Gurin [1929] and by Chick and Roscoe [1929] that even this more stable factor is destroyed by prolonged heating (4 to 6 hours) in the presence of alkali.

Another fact illustrated by the above curves is that at the beginning of the experiment the storage period for vitamin B_3 is approximately 5 weeks, while that for vitamin B_2 is about 3 weeks at the most. Once the store of vitamin B_3 has been lowered and the daily doses substituted, an omission of vitamin B_3 stops growth at the end of one week (see rats 279, 280 and 282).

SUMMARY.

Further evidence is produced for the existence of a third "B" factor. This factor has now been concentrated and obtained free from other factors.

In conclusion, I wish to thank Professor R. A. Peters for his helpful advice and criticism throughout this work, and the Medical Research Council for a personal grant.

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XII. URICASE AND ITS ACTION.

By STANISLAUS JOHN PRZYLECKI.

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(*Received December 30th, 1929.*)

In a previous communication [Przylecki, 1928] various methods for the preparation of uricase were given. Later work has shown that the uricolytic activity of those preparations purified by dialysis in the presence of such antiseptics as thymol, recommended by previous workers in this field, is due to bacterial action. In view of this, all methods of preparation of uricase described in this paper, with the exception of the original glycerol-chloroform extract, which has been found to be sterile, must be regarded as being useless. Similarly, all conclusions drawn from experiments dealing with the uricolytic action of such extracts as contain bacteria (*i.e.* all but the original glycerol-chloroform extract) are invalid.

A detailed description of the micro-organism responsible for uricolysis in the above experiments will be published later and work is now in hand on the bearing of the discovery of bacteria in such extracts on earlier work on uricase.

REFERENCE.

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XIII. THE ANTISCORBUTIC POTENCY OF APPLES.

By MARY FORREST BRACEWELL, EDWARD HOYLE
and SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute, London.

REPORT TO THE MEDICAL RESEARCH COUNCIL.

(*Received December 31st, 1929.*)

INTRODUCTION.

It is not unreasonable to suppose that the vitamin C content of a fruit is controlled by a number of physiological factors and, consequently, that the minimum protective dose for a particular fruit should vary in accordance with some of its characteristics. That being so the establishment of a definite relationship between the antiscorbutic activity and the physiological condition of a plant would in no small measure contribute to our knowledge of the character of a vitamin and might even reveal its identity. It has been already pointed out [Zilva, 1927, 1928] that further progress by chemical purification must be slow, since, presumably, the removal of "impurities" beyond a certain stage brings about a degree of instability in the vitamin which makes it almost impossible to ascertain its presence by biological tests. An indirect approach on the above lines to the subject seems, therefore, to be justified at this stage of the study of the antiscorbutic factor.

This communication is devoted to exploratory experiments designed to detect the relations, if any, between the antiscorbutic activity of apples and the variety, soil, age and conditions of storage. The effect of heating on antiscorbutic activity is also discussed. It may be considered to be only preliminary in character, since, if a connection between the antiscorbutic activity of the apple and one of its physiological functions is to be definitely established, it must be done by a process of elimination. This procedure is, however, complicated by the inter-relation of a number of factors and, further, by technical difficulties arising from the seasonal character of the experimental material and the long duration and laboriousness of the tests.

It was essential in an investigation of this character to attain the highest degree of accuracy in order to establish reliable quantitative criteria. Standardised conditions, which necessitated the use of a great number of animals, were therefore observed. Only general results are given in this paper. Full details of these experiments will be communicated in due course in a special report to the Medical Research Council.

HISTORICAL.

Holst and Frölich [1912] found that out of four guinea-pigs kept on a diet of rye bread baked with yeast, supplemented with 30 g. of raw apple daily, three succumbed on the 39th, 51st and 52nd days respectively—the last two showed symptoms of scurvy at the *post-mortem* examination. The fourth animal was still alive after 87 days' subsistence on this diet. They conclude that the apple is not a good source of the antiscorbutic factor.

Robison [1919] investigated the antiscorbutic activity of concentrated apple juice, prepared by the "Kestner evaporator." In this process the duration of heating is less than one minute and the maximum temperature attained is about 102° F. The jelly which was tested contained about 83 % of total solids and a daily dose of 4 g. of this concentrated juice (equivalent to 24 g. of pressed juice) protected guinea-pigs weighing 400 g. from scurvy; a daily dose of 1 g. of this concentrated juice was barely sufficient to afford complete protection, whilst one of 0·5 g. delayed the onset of scurvy for only a short time.

The British Mission sent to Vienna soon after the war [Chick, 1920] observed that 50 g. of raw apple juice administered daily as an antiscorbutic failed to prevent the onset of early symptoms of scurvy in children.

Givens, McClugage and Van Horne [1922] protected guinea-pigs from scurvy with daily doses of 10 g. of raw apple juice. When the raw apple was subjected to any considerable heating, such as is ordinarily employed in preservation by desiccation or canning, the vitamin in the apple was considerably inactivated. Dried apple peelings were found by these authors to be active in daily doses of 2 g. (22·26 % of original weight).

Kohman, Eddy and Carlsson [1924] administered 5, 6 and 7·5 g. daily of raw, cold-stored, apple to guinea-pigs. The animals on all the doses were losing weight on the 20th day of the experiment. The stored apples were, therefore, replaced at this stage by fresh, early summer apples, purchased in the open market. This produced considerable improvement in the animals, although the above doses of the fresh apple were inadequate to protect guinea-pigs from scurvy. The authors consider this observation as indicating that raw apples in storage gradually lose their antiscorbutic potency. These workers have further found that apples kept in 2 % salt solution for about 18 hours before processing retained their original antiscorbutic activity; on the other hand, some other methods of canning had a deleterious effect on the antiscorbutic activity of the fruit.

THE ANTISCORBUTIC ACTIVITY OF DIFFERENT VARIETIES OF APPLES.

The following English varieties, from different seasons and grown on different soils, were investigated: Bramley's Seedling (cooking variety), Worcester Pearmain (dessert variety), Cox's Orange Pippin (dessert variety), Woodbine (cider variety), Dabinett (cider variety) and King Edward (cooking variety). Various imported varieties were also investigated and will be discussed later.

The results are summarised in Table I. Bramley's Seedling stands out as markedly the most active of the varieties tested. The high antiscorbutic content was established in these apples coming from different parts of England and picked at different seasons, and, therefore, it is unlikely to be accidental. The other cooking variety, King Edward, which resembles the Bramley's Seedling in many respects, is very much less active. The next in order of activity is the Dabinett, but when compared with Bristol Bramley's grown in an adjacent orchard on a more or less similar soil, it is between three or four times less active. Of the eating varieties, Cox's Orange Pippin is about twice as active as the Worcester Pearmain, but they both fall far below Bramley's Seedling in activity.

Table I. *Relative antiscorbutic potency of English varieties.*

Dose g.	Normal Bramley 1927, Canterbury	Normal Bramley 1928, Burwell	Normal Bramley 1928, Bristol	Worcester Pearmain 1928, Burwell	Normal Cox's Orange Pippin 1928, Burwell	Woodbine 1928, Bristol	Dabinett 1928, Bristol	King Edward 1928, Burwell
3	+ + +	+ + + ±	+ + + +	No protection	±	No protection	No protection	No protection
5	+ + + +	+ + + +	+ + + +	,,	+	?	+ + ±	,,
10	+ + + +	+ + + +	+ + + +	±	+ + ±	+	+ + + +	±
20	+ + + +	Not tested	+ + + +	+ + +	+ + + ±	+ + + +	+ + + +	+ +

It has been found that there was no relationship between the age of the tree from which the apples were picked and the antiscorbutic activity. The Bristol Bramley's Seedlings were picked from trees eight years old and the Canterbury and Burwell Bramley's Seedlings from trees about twice this age, yet the difference in the antiscorbutic activity of these apples is not significant. On the other hand, all the Burwell apples (Cox's Orange Pippin, Worcester Pearmain and Bramley's Seedling), differing so much in activity, came from trees of approximately the same age. Nor can there be a direct relationship between the soil on which the apples are grown and their vitamin content, since the Bramley's Seedlings represent three different types of soil, whilst the Burwell and Long Ashton apples which showed a varying vitamin content came from similar soils.

THE ANTISCORBUTIC ACTIVITY OF EARLY GATHERED APPLES.

These experiments were not instituted with the intention of making a detailed study of the relationship between the varying stages of the age cycle on the antiscorbutic activity of the apple. Such an inquiry is in progress. They were intended to be more of an exploratory character in order to ascertain whether a very marked difference in activity is obtained by picking apples earlier. The experiments were carried out on the Canterbury Bramley's Seedling and the Burwell Cox's Orange Pippin. Apples of these varieties were picked from the same trees 14 days before the normal apples were gathered. It will be seen from Table II that there is no marked difference between the activity of these apples and that of the normal picking from the same trees a fortnight later. Nor is there to be observed a marked difference in the activity in the case of the Cox's Orange Pippin.

Table II. *Relative antiscorbutic potency of early and normal apples.*

Dose g.	Early Bramley 1927, Canterbury	Normal Bramley 1927, Canterbury	Early Cox's Orange Pippin 1928, Burwell	Normal Cox's Orange Pippin 1928, Burwell
3	+ + + ±	+ + +	±	±
5	+ + + ±	+ + + +	+	±
10	+ + + +	+ + + +	+ ±	+ + ±
20	+ + + +	+ + + +	Not tested	+ + + ±

INFLUENCE OF STORAGE ON THE ANTISCORBUTIC POTENCY OF APPLES.

Preliminary observations were made on the vitamin C in apples which aged after gathering under two empirical methods of storage, namely, "cold storage" and "gas storage." In the former case the apples were kept at 1° (constant) in the air and in the latter in an atmosphere of 10 % carbon dioxide, 11 % oxygen and 79 % nitrogen at 10°. The storing was done at the Low Temperature Research Station, Cambridge.

Table III shows that little deterioration had taken place in the vitamin during storage and that the loss, such as it was, was more marked in the "gas-stored" apples. This disparity in the inactivation of the vitamin is particularly marked in the Cox's Orange Pippin. The question as to what extent it is characteristic of this variety cannot at present be answered, since only one storage experiment has so far been carried out with it. It is further seen that early picking and soil do not seem to influence the behaviour of the antiscorbutic factor in apples on storage.

Table III. *Relative antiscorbutic potency of stored apples.*

Dose g.	Early Bramley 1927, Canterbury	Early Bramley 1927, Canterbury. Cold-stored 1°, 98 days	Early Bramley 1927, Canterbury. Gas-stored 10°, 98 days	Normal Bramley 1927, Canterbury	Normal Bramley 1927, Canterbury. Cold-stored 1°, 98 days	Normal Bramley 1927, Canterbury. Gas-stored 10°, 98 days
3	+ + + ±	+ + +	+ ±	+ + +	+ + ±	+ ±
5	+ + + ±	+ + + ±	+ + +	+ + + +	+ + +	+ + + ±
10	+ + + +	+ + + +	+ + + ±	+ + + +	+ + + +	+ + + +
20	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +

Dose g.	Normal Bramley 1928, Burwell	Normal Bramley 1928, Burwell. Cold-stored 1°, 105 days	Normal Bramley 1928, Burwell. Gas-stored 10°, 140 days	Cox's Orange Pippin 1928, Burwell	Cox's Orange Pippin 1928, Burwell. Cold-stored 1°, 79 days	Cox's Orange Pippin 1928, Burwell. Gas-stored 10°, 83 days
3	+ + + +	+ + + ±	+ + +	±	No protection	No protection
5	+ + + +	Test not finished	+ + + ±	+	±	„
10	+ + + +	„	+ + + +	+ + ±	+ + +	„
20	Not tested	Not tested	+ + + +	+ + + ±	+ + + +	±

THE ANTISCORBUTIC ACTIVITY OF IMPORTED APPLES.

The following imported varieties have been tested: *Cleopatra* Louden, Western Australia (May, 1927) and Karragullen, Western Australia (April, 1928), *Jonathan* (May, 1927), *Strawberry Pearmain*, Louden, Western Australia (May, 1927), *Cleopatra*, Baghdad, Tasmania (May, 1927) and New Zealand (May, 1927), *Kings*, Ontario (November, 1927) and Burlington, Ontario (January, 1929), *Kings*, Nova Scotia (November, 1927), *Jonathan*, British Columbia (November, 1927), Penticton, British Columbia (October, 1928) and *Cox's Orange Pippin*, Penticton, British Columbia (October, 1928).

Circumstances did not favour the collection of as many details about the imported experimental material as was possible in the case of the English apples but it will be seen from the above that the general aim was to get (1) a number of varieties, (2) one variety from different places and (3) one variety from as nearly as possible the same place two consecutive seasons.

Table IV. *Relative antiscorbutic potency of imported dessert apples.*

Dose g.	Cleopatra 1927, Australia	Cleopatra 1928, Australia	Jonathan 1927, Australia	Strawberry Pearmain 1927, Australia	Cleopatra 1927, Tasmania	Cleopatra 1927, New Zealand
3	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
5	No protection	No protection	No protection	?	No protection	No protection
10	?	,,	?	±	± ?	± ?
20	± ?	+ +	+	+ ?	+ +	+

Dose g.	Kings 1927, Ontario	Kings 1928, Ontario	Kings 1927, Nova Scotia	Jonathan 1927, British Columbia	Jonathan 1928, British Columbia	Cox's Orange Pippin 1928, British Columbia
3	No protection	Not tested	No protection	No protection	No protection	No protection
5	±	No protection	,,	±	±	± ?
10	+ + +	+ ?	+	+ +	+	± ?
20	+ + + +	Not tested	+ + + ±	+ + + +	+ + +	+ ±

It is seen from Table IV that the Australian and the New Zealand apples were not as potent as the Canadian apples, which were almost as potent as the English dessert varieties. On the other hand, there is comparatively little difference in activity among the different varieties coming from various places in the same country and belonging to two seasons, which is consistent with the observations made on the English apples. How can one explain the difference in the activity of the Australian, Canadian and English apples? In view of the results obtained in the storage experiments it would seem that this disparity is most probably due to the different lengths of time that elapse between the picking and the testing of the apples. It is to be noted that the Ontario King apples (January, 1929), which could only be tested in 5 g. and 10 g. doses, about two months later than the majority of the Canadian apples showed, as far as one can judge, from this incomplete test, a vitamin content roughly of the order of the Australian apples and were decidedly lower in potency than the same variety of the previous year tested earlier (November, 1927). Another point of interest is that British Columbia Cox's Orange Pippin, although tested early in October, shows a lower potency than the other varieties of the same origin. Unfortunately, the test, to some extent, was marred by the fact that most of the test animals on the highest dose did not consume their entire 20 g. of apple. The result, however, indicates the possibility that the antiscorbutic activity of Canadian Cox's Orange Pippin as in the case of this English variety, has a tendency to deteriorate more quickly than that of other varieties.

The effect of heat on the antiscorbutic activity of Bramley's Seedling.

As Bramley's Seedling is a cooking variety, it was of interest to ascertain the influence of heat on its antiscorbutic activity. The potency of heated apples before storage, after cold storage and after gas storage was, therefore, investigated. It was advisable to study the effect of heat on stored apples since evidently the stability of vitamin C is controlled by other factors than the vitamin itself [Zilva, 1927, 1928] and consequently there was the possibility that observations made on unstored apples might not hold true of apples which had undergone changes on storage.

The apples were placed, in their skins, in a drying oven previously brought up to 160°. On the introduction of the apples the temperature fell to about 115°, at which temperature the heating continued for about 50 minutes; by this time the flesh of the apples was quite soft. On several occasions observation was made on the temperature of the apple by introducing a thermometer into the tissue to the depth of the core. The temperature rose from about 25° after 5 minutes to about 95° at the end of the heating. After cooling the soft pulp was scooped out and administered to the guinea-pigs in equivalent doses of the raw apple (there was usually a loss of about 10 % of the original weight in the process of heating). The Burwell Bramley's Seedling (October, 1928) were used in these tests. Results of the experiments (Table V) show that very little destruction of the vitamin took place during the heating, whether freshly picked, cold-stored or gas-stored apples were used.

Table V. *Relative antiscorbutic potency of heated apples.*

Dose g.	Normal Bramley 1928, Burwell	Heated normal Bramley 1928, Burwell	Normal Bramley 1928, Burwell. Cold-stored 1°	Heated normal Bramley 1928, Burwell. Cold-stored 1°	Normal Bramley 1928, Burwell. Gas-stored 10°	Heated normal Bramley 1928, Burwell. Gas-stored 10°
3	+ + + +	+ + + +	+ + + +	+ + + + ?	+ + +	+ + +
5	+ + + +	+ + + +	Test not finished	+ + + +	+ + + +	+ + +
10	+ + + +	+ + + +	,,	+ + + +	+ + + +	+ + + +
20	Not tested	+ + + +	Not tested	+ + + +	+ + + +	+ + + +

THE CHEMICAL COMPOSITION OF THE APPLE VARIETIES TESTED.

The biological test for vitamin C yields, of course, the composite effect of the batch as a whole. It gives no information concerning the individual variation of the apples in antiscorbutic potency. Under these circumstances, in order to obtain a fair comparison between certain chemical data and the vitamin content of apples, the material to be tested should be in a stable and uniform condition which would represent both chemically and biologically the average of the batch. An elaboration of such a method is in progress. In order, however, to obtain some information about the chemical composition of the fruits tested, a few representative apples of each batch were analysed. Although the number of estimations of each sample of apples was too few for the formation of any definite conclusions, nevertheless, the data offered some information which at this stage of the inquiry may be considered of an indicative nature.

Figures more or less similar to those previously obtained by other workers for these varieties have been recorded and afford no information which can be correlated with the biological activity. Particularly striking was the similarity in the general chemical composition of the Bramley's Seedling and the King Edward, which differ so much in their vitamin content. There was one chemical dissimilarity, however, namely that of the nitrogen content. Unfortunately, owing to an oversight, the nitrogen of only two batches was determined, namely, those of the gas-stored Burwell Bramley's Seedling (1928) and of the stored King Edward (1928), which were carried out on three representative apples of each batch in March, 1929. The percentages of N were as follows: King Edward, 0·061, 0·070, 0·072; Bramley's Seedling, 0·027, 0·035, 0·038. In a work of this nature these figures are too few for drawing categorical conclusions but they are, nevertheless, significant enough to be considered in future research.

CONCLUSIONS.

One of the main objects of this investigation was to ascertain whether a functional relationship existed between the antiscorbutic principle and any other factor or factors in the apple. The experimental evidence so far obtained does not supply the answer. Considering the number of variables involved, it would indeed have been a very fortunate coincidence if the first set of preliminary experiments were to have supplied definite information. We have, nevertheless, made a number of observations, some of which may be considered as more or less definite, whilst others can serve at this stage only as indications. If some of these observations are not capable of full interpretation at present, their significance will, in all probability, be disclosed as the investigation develops.

It is no surprising feature to find a variation in the antiscorbutic activity of apples, but the persistent high vitamin content of the Bramley's Seedlings attracts special attention. Judging from the results so far obtained one may exclude age of tree, soil and season as being directly responsible for this high activity, since, as we have seen, the Cox's Orange Pippins, Worcester Pearmains and Dabinetts were grown under similar conditions and yet were less active. The contrast is even more marked when the Bramley's Seedlings are compared with the King Edwards, a variety resembling them so much. Apart from the difference in the vitamin C content the only disparity which was observed in these two varieties was in the nitrogen content, but, as already stated, the number of estimates was few. Whether this property of the Bramley's Seedling is "racial" and if so, whether it is associated with any other outstanding characteristic, has still to be established. It would be further of interest to ascertain whether the smaller degree of difference in the antiscorbutic activity observed among the dessert and cider varieties is definitely characteristic of these apples.

That high antiscorbutic activity is the property of the average Bramley's Seedling, whether freshly picked or stored and that it persists after heating, is a point of some interest to the dietetician. Yet one can hardly consider the lesser antiscorbutic activity of the dessert varieties, English or imported, as inimical to their nutritive value, since in the consumption of these varieties under normal conditions the vitamin C requirements are usually amply covered by the general diet; much rather is the dessert apple valued for its palatability, flavour and other characteristic properties. This point is stressed owing to the fact that there is a tendency at present to give wrong values to experimental facts obtained in vitamin research when applied to practical hygiene and commerce.

It is also quite difficult at this stage to interpret the significance of the disparity in activity of the apples which were stored at 10° in an atmosphere of oxygen, carbon dioxide and nitrogen and of those which were allowed to age in the ordinary atmosphere at 1°. What bearing, if any, vitamin C has on the physiological changes of the apple in its progress through youth, maturity and senescence is a problem which still awaits solution and which we hope will be elucidated by the experiments which are now in progress.

SUMMARY.

1. Of a number of apple varieties tested for their antiscorbutic potency, Bramley's Seedling was found to be markedly more active than all the other varieties, which differed among themselves comparatively very much less in their vitamin C content. There were no indications that the character of the soil, the age of the tree or season had any bearing on the antiscorbutic activity of the apple.

2. Bramley's Seedlings picked from the same tree 14 days before the normal crop were approximately of the same antiscorbutic activity.

3. There was little loss in the vitamin C content of apples stored at 1° in the air or at 10° in a mixture of carbon dioxide, nitrogen and oxygen for about

3 months. The gas-stored apples showed, however, a definitely greater deterioration in the vitamin.

4. Tests carried out on a number of imported dessert apples showed that the activity was higher in those cases in which the time elapsing between the picking of the fruit and the testing was the shortest. There was also no indication in the case of these apples of any very marked difference in activity which could be correlated with the difference in variety.

5. Heating of Bramley's Seedlings in their skins hardly affected their antiscorbutic activity.

It is with great pleasure that we take this opportunity of acknowledging our indebtedness to Drs. West and Kidd of the Low Temperature Research Station, Cambridge, and Professor Barker of the Agricultural and Horticultural Research Station, Long Ashton, for much valuable help and advice. It is evident from the nature of the inquiry that without their assistance the pomological aspect of this investigation would have been considerably restricted.

Our thanks are also due to Mr P. G. H. Barter of the Empire Marketing Board for his assistance in collecting the imported varieties of apples.

Having had the privilege of consulting Sir William Hardy at various stages of this inquiry we cannot conclude without expressing our high appreciation of his stimulating encouragement.

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XIV. OBSERVATIONS UPON THE REDUCING SUBSTANCES OF PIGEONS' BLOOD.

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It has been observed from time to time by various investigators, *e.g.* Funk and v. Schönborn [1914], Funk [1920], Honeywell [1922], Riddle and Honeywell [1923], Collazo [1923], Randoin and Lelesz [1925], Marrian, Baker, Drummond and Woollard [1927], Schwarz and Heinrich [1928], and Kinnersley and Peters [1929], that the total reducing value of normal avian blood is considerably higher than that of normal mammalian blood; in fact this value, when expressed in terms of glucose, seems to show that the sugar level in normal birds is roughly the same as that which is characteristic of human diabetics. Schwarz and Heinrich, for example, found that the average total reducing value of hens' blood was as high as 253 mg. of glucose per 100 cc. when estimated by the Hagedorn and Jensen method. Marrian *et al.* obtained values of 180 mg. per 100 cc. for pigeons deprived of food for 24 hours. Collazo found 210 mg. of glucose per 100 cc. for pigeons; and Riddle and Honeywell observed variations in the sugar level according to the breed of pigeons used, their values lying between 149 and 258 mg. of glucose per 100 cc. The question therefore arises whether these high values are caused by an additional amount of glucose as compared with that of mammalian blood, or whether the non-glucose reducing substances in avian blood are present in amounts sufficient to lower the true glucose value to one approaching mammalian limits. Intimately connected with this problem are the unpublished observations (*a*) by Kinnersley and Peters, that the reducing value of the blood of pigeons in insulin convulsions is about 70 mg. of glucose per 100 cc. when estimated by the Hagedorn and Jensen method; and (*b*) by J. T. Irving, that the apparent glucose content of pigeons' blood is about 60 mg. when glycolysis has proceeded until the reducing value has become constant. We take this opportunity to express our indebtedness to Dr. Irving for allowing us to mention his observations. These results seem to show that the amount of non-glucose reducing substances in pigeons' blood is considerable, and it is therefore important to ascertain their nature, relative proportions and, if possible, their function. This information is needed in order to decide what is the best method of estimating the blood-"glucose," but in addition unusual interest centres round the question of function, since it has been shown by Rüter [1923] that glycolysis of goose and hen blood is slight or absent *in vitro*, and by Bornstein and Ascher [1926] that this is due to the presence of oxygen, observations which we have confirmed in the case of pigeons. It was considered possible that the inhibition of glycolysis and the apparent high content of non-glucose substances might be interdependent.

EXPERIMENTAL.

The reducing substances of normal pigeons' blood.

It is evident that the solutions of the two problems outlined above may be approached by the same route, namely by determining the total "sugar," eliminating the true glucose by means of insulin or glycolysis, and then estimating the substances responsible for the residual reduction. This choice of method of eliminating glucose is justified by the work of Hiller, Linder and Van Slyke [1925], who concluded that glycolysis and the administration of insulin resulted in the destruction of the same amount of glucose. From the figures thus obtained the true glucose content may be calculated. In order to avoid any criticism which might be levelled at this statement, we wish to make it clear that we do not contemplate entering the present controversy on true blood-glucose. For the purpose of the present communication, we shall consider as the blood-glucose the reducing substance (or substances) which disappear during glycolysis or after insulin treatment.

Throughout this work the reducing values have been determined by the Hagedorn and Jensen method. A survey of the literature on the subject of blood-sugar has convinced us that it would be unwise to base conclusions on results derived by different methods. The work of Herbert and Groen [1929] is of special interest in this connection.

The samples have been collected by stunning and guillotining the pigeons (in some cases stunning was omitted), and shedding the blood directly into heparin (6 mg. per bird). This anti-coagulant has no significant reducing value in the Hagedorn and Jensen estimation. Assuming that it survives completely the initial stage of protein precipitation, its presence in a concentration of 1 mg. in 1 cc. of blood would raise the blood-"sugar" by 1–2 mg. per 100 cc. since 0·5 mg. of heparin is equivalent to 0·04 cc. of 0·005 N alkaline ferricyanide.

Glycolysis, though absent in aerated avian blood, proceeds normally in presence of potassium cyanide or in absence of oxygen [Bornstein and Ascher, 1926]. In the glycolytic experiments recorded in this paper, it has been found advisable to add 1 mg. of potassium cyanide per cc. of blood. In this concentration potassium cyanide has no significant effect in the Hagedorn and Jensen method, supposing that it survives the initial protein precipitation, since determinations with aqueous solutions have shown that 0·1 mg. of potassium cyanide is equivalent to 0·01 cc. of 0·005 N alkaline potassium ferricyanide.

Characteristic curves are given in Fig. 1 for the glycolysis in presence of cyanide of normal pigeons' (A) and of cockerels' blood (C) when agitated gently at 37°. Curve A shows that, in confirmation of Irving's [1926] observations for mammalian blood and unpublished observations for pigeons' blood, glycolysis proceeds in an approximately linear manner. After some 6 hours in pigeons' blood, it stops rather abruptly at 60–70 mg. "apparent" glucose per 100 cc. This value has been regarded throughout this research as the "residual" reduction after glycolysis. Curve C shows that in a cockerel glycolysis stops abruptly at approximately the same level and indicates that the facts here described are also true for hen blood. This matter has not been further investigated.

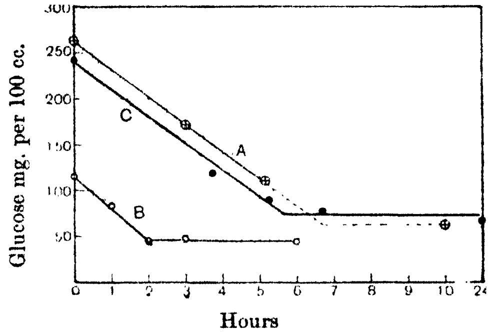

Fig. 1. Glycolysis in presence of cyanide.
A. Normal pigeon. B. Abnormal avitaminous pigeon. C. Normal cockerel.

The curve B represents the glycolysis of the blood of a polyneuritic pigeon under the same conditions, and is included in order to show that the form of the curve is unaltered (as compared with the normal bird). The values in curve B are however exceptional, being the lowest ever recorded for B-avitaminous pigeons in this laboratory. We have been unable to obtain curves for more typical avitaminous pigeons owing to an apparent inefficiency of the glycolytic system in such cases (see p. 101).

The total value of the "residual" reduction in terms of glucose.

In Table I may be seen the effect of eliminating the glucose from the blood of a number of typical normal pigeons. Attention is directed to the high total blood-"sugar" and the comparative constancy of the residual reduction figure after glycolysis.

Table I.

Results in mg. glucose per 100 cc. blood.

Bird	Initial "sugar"	Duration of glycolysis in hours	Residual reduction	
			KCN	Anaerobic
1 Insulinised	(88)*	4	69	71
2 Normal	212	—	—	—
3 ,,	237	10	74	78
4 ,,	239	24	68	—
5 ,,	223	12	82	—

* In convulsions.

The average value for the blood-"sugar" in normal pigeons as taken out of the cage (and therefore feeding) has been found to be 230 mg. ± 20 mg. per 100 cc., and that for birds kept without food for 24 hours 205 mg. ± 15 mg.

Since the residual reduction value represents non-glucose substances, the approximate glucose content of pigeons' blood may be derived from the preceding table. The average value for the total "sugar" is 228 and that of the residual reduction 73, whence the glucose content is 155 mg. per 100 cc. for the feeding bird, a value greater it is true than that of most mammalian bloods, but lower than the uppermost limit for the ox [Schwarz, 1928]. Hence the glucose value for the blood of pigeons deprived of food for 24 hours must be 135 ± 15 mg. per 100 cc. It is clear therefore that the residual reducing value of pigeons' blood is considerably higher than that of mammalian blood, and consequently the true glucose value of birds' blood is not so widely divergent from that of mammals as the Hagedorn and Jensen estimations appear to show [compare Lund and Wolf, 1926].

The nature of the residual reducing substances.

Attention was then directed to the nature of the substances responsible for the residual reduction, and uric acid, glutathione and ergothioneine came into consideration as possibilities. Uric acid has long been known to occur in avian blood, and Randoin and Fabre [1927] have detected aliphatic sulphydryl compounds in pigeons' blood and estimated the average content as 61 mg. per 100 cc. of blood, although individual variations were large. Hunter [1928] found ergothioneine in fowls' blood, and we have isolated some 5 mg. of the hydrochloride from the combined bloods of ten pigeons (see p. 101).

Reducing value of uric acid. Holden [1926] has observed that the reducing power of uric acid in the Hagedorn and Jensen method is 53 % of that of glucose. Flatow [1926] showed that uric acid reduces cold potassium ferricyanide solution. Similar experiments have now been performed from which it has been deduced that uric acid when oxidised by cold alkaline potassium ferricyanide solution has a reducing value of 53 % of that of glucose when oxidised by hot ferricyanide, and that this value remains unchanged when the mixture of uric acid and ferricyanide is heated as in the Hagedorn and Jensen method.

Oxidation by cold alkaline ferricyanide. Since the Hagedorn and Jensen method involves the liberation of free iodine, it is clear that compounds such as ergothioneine and glutathione, which contain sulphydryl groups, will have an "apparent" reducing value even if stable to ferricyanide, since the amount of ferricyanide reduced is measured by estimating by means of thiosulphate the amount of iodine liberated by the excess of ferricyanide. The utilisation of iodine in such oxidations would therefore appear as oxidation by ferricyanide. In the hope of estimating the non-glucose reducing substances by such means, a "cold Hagedorn and Jensen method" has been employed. The sole modification is that the alkaline ferricyanide solution and blood filtrate are not heated but are at once mixed with potassium iodide-zinc sulphate solution, then with acetic acid and titrated with thiosulphate. The blank estimation is of course a simple titration of unheated ferricyanide with thiosulphate. In Table II are recorded the "cold" reducing values, calculated as mg. glucose in 100 cc. of the bloods of the same pigeons used for the experiments of Table I.

Table II.

1 and 8 insulinised, 2–7 normal. Results in mg. glucose per 100 cc.

Bird	1	2	3	4	5	6	7	8
Initial cold value	32	34	32	—	32	11	30	44
After glycolysis	—	—	—	39	35	14	32	—

It is clear that with one exception, which will be discussed below, there is little significant variation in normal pigeons and that the "cold" value is not affected either by glycolysis in presence of cyanide or by the administration of insulin. It should also be noted that the figures represent only 40–60 % of the total non-glucose reduction. This fact will be referred to later.

Table III has been constructed to show that the substances responsible for the "cold" reducing value and also for the residual reduction by the customary method are present almost entirely in the corpuscles, and that the serum is almost free from these substances.

Table III.

Results in mg. glucose per 100 cc. Bloods centrifuged, relative volumes of corpuscles and serum noted, and plasma pipetted off. Corpuscles usually diluted to original volume of blood with saline. Figures in brackets are samples taken after complete glycolysis.

	Corpuscles		Serum		Whole blood	
Bird	Cold	Hot	Cold	Hot	Cold	Hot
1	34	73	4	—	34	213
2	—	50	—	(10)	—	368
3	—	71 (53)	—	(21)	—	370
4	—	(53)	—	(11)	—	(63)

Table IV.

Amounts in mg. Vol. of solutions, 15 cc.

Method	Ergothioneine added	Glucose added	Apparent glucose	Glucose calc.	Reducing power glucose = 100
Cold*	0·172	None	0·097	—	56
Cold†	0·178	„	0·099	—	56
Cold‡	0·200		0·108	—	54
Hot*	0·172		0·104	—	61
Hot†	0·178		0·117	—	66
Hot‡	0·200		0·133	—	66
Cold	None	0·299	None	—	—
Hot	„	0·312	0·229	0·312	—
Hot	0·086	0·149	0·200	0·180	59

* Ergothioneine from ergot.
† Ergothioneine from ergot (different sample).
‡ Ergothioneine from pigs' blood.

Reducing values of ergothioneine. Table IV shows the reducing power of ergothioneine by the "hot" and "cold" Hagedorn and Jensen methods and makes it clear that coupled oxidations with glucose do not occur (cf. Somogyi, 1927]. Holden [1926] has shown that uric acid, cystine, creatinine and amino-acids do not give rise to coupled oxidations. Sjollema [1927] states that ergothioneine in 0·1 % solution has a reducing value of 84 as compared with glucose 100.

Reducing values of glutathione. Table V shows the "hot" and "cold" values for the tripeptide reduced glutathione [Hopkins, 1929].

Table V.

Amounts in mg. Vol. of solutions 15 cc.

Exp.	Method	Glutathione added	Apparent glucose	Reducing power glucose = 100	Remarks
1	Cold	0·500	0·088	17·6	Titrated immediately (30 secs.) after adding alkaline ferricyanide.
2	Cold	0·500	0·064	12·8	Titrated 5–15 mins. after adding alkaline ferricyanide
3	Hot	0·500	0·227	45·4	-

Exps. 1 and 2 show that the speed of estimation makes a considerable difference to the result obtained by the cold method, and Exp. 3 that hot ferricyanide gives a much higher value than cold.

Estimations of non-glucose reducing substances in different filtrates.

In attempting to draw up a balance sheet of non-glucose reducing substances, we were led to examine qualitatively, and later quantitatively, blood-filtrates obtained by different methods. The following table gives a list of the compounds present.

Table VI. *Reducing compounds other than glucose present in blood-filtrates.*

Total: 70 mg. ± 10 mg. per 100 cc. (calculated as glucose).

	Substance	Tungstic acid	Trichloroacetic acid	Zinc	Approx. amount in zinc filtrate in mg. per 100 cc.
(1)	Uric acid	+(*a*)	?	?	?
(2)	—SH compounds	+(*b*)	+(*e*)	—	—
(3)	—S.S— compounds	+(*c*)	+	—	—
(4)	Ergothioneine	+(*d*)	?	+(*f*)	30
(5)	Unknown, reducing cold ferricyanide	?	?	+	10
(6)	Unknown, reducing hot ferricyanide	?	?	+	40

(*a*) Benedict [1922]. (*b*) Nitroprusside reaction. (*c*) Walker's reaction [1925]. (*d*), (*f*) Compounds giving G. Hunter's ergothioneine reaction [1928]. (*e*) Less —SH compounds present than in (*b*).

It will be seen that the zinc filtrates (Hagedorn and Jensen) are freer than others from all compounds except ergothioneine, and that for any given blood the variations between the reducing values obtained upon a zinc filtrate and a trichloroacetic acid filtrate will depend upon fluctuations in ergothioneine content and the extent to which other sulphur compounds are present in the trichloroacetic acid filtrate. This accounts for results of Kinnersley and Peters, in which with pigeons' blood they have found irregular variations between the "sugar" values of these two filtrates respectively, the trichloroacetic acid filtrates being 20–30 mg. higher. Ergothioneine cannot be estimated in the trichloroacetic acid filtrates because this reagent inhibits the diazo-reaction even in neutralised filtrates.

After these experiments were completed, we became aware that Somogyi [1929, 1] had stated that ergothioneine, glutathione and uric acid are absent from zinc filtrates. The discrepancies between the two series of results are doubtless due to the fact that Somogyi precipitated the proteins by shaking at room temperature and not by heating, as in the Hagedorn and Jensen method. The present observations explain the statement by Somogyi in another paper [1929, 2] that the Hagedorn and Jensen technique leaves only about half as much of non-fermentable reducing substances in the filtrate as the tungstic acid procedure.

Bearing in mind that the cold reducing value of uric acid is 53 %, of ergothioneine 56 %, and of glutathione 17 % of that of glucose, we may now compare the amounts of ergothioneine, uric acid and aliphatic —SH compounds as estimated by accepted methods with the values obtained for the cold reduction of various types of filtrate.

Table VII.

The pigeons are the same as those in Tables I and II. Results in mg. per 100 cc. blood. 5–7 normal, 8 insulinised.

			Tungstic acid			
Bird	Zinc. Ergo-thioneine	Trichloro-acetic acid. Total —SH as cysteine	Total —SH as cysteine	Indirect uric acid	Rockwood thioneine as uric acid	Ergo-thioneine
6	0	42	61	3·8	1·0	0
7	33	61	85	3·7	1·3	13
5	—	85	—	4·5	1·7	—
8	—	—	—	—	—	10
17	30	—	—	—	—	6
18	27	—	—	—	—	5

Ergothioneine and —SH compounds. Table VII records estimations of these in the bloods of normal pigeons. Ergothioneine was estimated by Hunter's method, using aqueous solutions of ergothioneine hydrochloride as standards, and the total sulphydryl compounds were determined by adding to the solutions an excess of 0·005N iodine in potassium iodide solution and titrating the unused portion with 0·005N thiosulphate solution using starch as indicator. Tungstic acid filtrates were rendered faintly acid before the estimation of sulphydryl compounds. The term "indirect uric acid" has the meaning given by Bulmer, Eagles and Hunter [1925] and represents that portion of the silver precipitate which is decomposed by sodium chloride-hydrochloric acid solution and considered to be true "uric" acid. "Rockwood thioneine" represents the insoluble solid from this treatment, which was regarded as ergothioneine by Rockwood, Turner and Pfiffner [1929]. This fraction contains a considerable amount of aliphatic sulphydryl compounds or disulphides as may be shown by means of Walker's test, and the figures have no real significance. The "indirect uric acid" fraction also contains traces of these compounds, but when estimated colorimetrically their concentration was about 1 in 8000. Their presence may be neglected, since we have found that one part of uric acid gives the same colour as 125 parts of cystine in the Benedict method [1922] of estimation.

It will be noticed that whereas zinc filtrates tend to give 30 mg. ergothioneine (with exception of bird 6 to which reference is made later), the values for ergothioneine in the tungstic acid filtrate are lower and variable. This is in part due to the difficulty of comparing the colours in the latter filtrates. In the case of 17 and 18, the tungstic acid filtrates gave a colour with Hunter's reagent which matched phenol red of p_H 7·3 and 7·1 respectively, and the values were obtained by matching against a phenol red standard at p_H 7·1. For this purpose we made the assumption that there was present in the p_H 7·1 solution 1/5 phenol red dissociated as the alkaline indicator. The results for the tungstic acid filtrates can be regarded as only approximate, but it is clear that zinc filtrates appear to contain considerably more substances which give Hunter's ergothioneine reaction than do tungstic acid filtrates. These may be ergothioneine itself, or perhaps other thiolglyoxalines, since it is stated [Benedict and Newton, 1929] that tungstic acid coagulation precipitates a large proportion of the ergothioneine present in blood. In this connection it may be noted that Sjollema [1927] concluded from sulphur analysis that sulphur-containing compounds other than ergothioneine and glutathione are present in blood.

Hot and cold Hagedorn and Jensen estimation of different types of filtrate.

In Table VIII are shown the results of estimations made on the zinc, trichloroacetic acid and tungstic acid blood-filtrates of the same pigeons by the cold and hot Hagedorn and Jensen methods. Though not numerous these results are sufficient to show the type of variation to be expected in general; the "sugar" values of zinc and trichloroacetic acid filtrates differ somewhat by the hot method, the latter being slightly higher, and the results for all three types of filtrate show variations by the cold method. These discrepancies are no doubt due in part to the fluctuating content of aliphatic sulphydryl compounds. Sjollema [1927] has observed a similar increase in non-glucose reducing substances in tungstic as compared with trichloroacetic acid filtrates. In view of the results with pigeon No. 6 it is interesting that Herbert and Groen [1929] have observed that the hot Hagedorn and Jensen method gives figures which are on the average 27 mg. per 100 cc. higher in tungstic acid than in zinc filtrates obtained from human blood.

It will be noted that "cold" values upon the tungstic filtrate give about 30 mg., or approximately the same as the zinc filtrate, though it must be emphasised that the substances responsible are different in the two cases.

Table VIII.

Results as mg. glucose per 100 cc. blood.

	Zinc		Trichloroacetic acid		Tungstic acid.
Bird	Cold	Hot	Cold	Hot	Cold
7	30	227	14	—	—
6	11	253	19	—	34
4	—	239	28	246	—
5	32	223	28	225	—
17	—	—	—	—	31
18	—	—	—	—	30

All trichloroacetic acid filtrates were neutralised before estimation.

Unknown substances reducing "cold" ferricyanide.

Special interest attaches to the estimation of zinc filtrates by the cold method because the amounts of non-glucose substances there present are unable to account for the whole of the reduction. This is best illustrated by reference to pigeon No. 6, from whose blood ergothioneine was absent. The cold value is still 11 mg. of glucose per 100 cc., and the uric acid content (estimated from the tungstic acid filtrate) is of the normal order. Assuming that the same amount is present in the zinc filtrate, it is approximately equivalent to 2 mg. of glucose, so that the equivalent of 9 mg. of glucose per 100 cc. remains unaccounted for. The assumption made here is probably unjustified if interpreted too exactly, but the uric acid content of zinc and tungstic acid filtrates would presumably be of the same order. Now uric acid has half the reducing power of glucose, and creatinine and creatine, which may also be present, are stable to cold alkaline ferricyanide in amounts representing 200 mg. per 100 cc. of blood, whereas Scheunert and Pelzchrzim [1923] have estimated the combined creatine and creatinine as only 4·7 mg. of creatinine per 100 cc., a figure in harmony with the results obtained for other avian bloods by different workers [Hunter, A., 1928]. It follows therefore that zinc filtrates must contain at least one other non-glucose reducing substance which represents about 10 mg. of glucose and is oxidised by cold alkaline potassium ferricyanide or by iodine. This conclusion is supported by the case of bird No. 7 where an approximate balance-sheet for the cold reduction value shows that the equivalent of some 10–12 mg. of glucose is again unaccounted for. Evidence leading to the same conclusion may be derived from a consideration of the cold reducing value for the avitaminous pigeon No. 14, namely 18 mg. of glucose per 100 cc., since ergothioneine was present only in small amount in the zinc filtrate (approximately 8 mg. per 100 cc.).

Unknown substance reducing hot ferricyanide.

Turning now to the residual non-glucose reduction of zinc filtrates by the hot Hagedorn and Jensen method as determined by insulin and glycolysis, the average increase of this over the cold values is 40 mg. of glucose per 100 cc. Part of this increase is no doubt due to creatinine, stated by Holden

[1926] to have 67 % of the reducing power of glucose and by Holmes and Holmes [1926] to have a maximum effect of about the same value. Part corresponds to creatine, which Holmes and Holmes have shown to have a maximal efficiency of about 25 % of that of glucose; and part must represent the increase in the "hot" value over the "cold" for ergothioneine. These corrections cannot amount to more than 3 mg. per 100 cc. in all, and it is therefore evident that a considerable balance remains unassigned, although its nature must at present remain indefinite. Three hypotheses, however, may be cited. The presence of a uric acid-ribose compound such as that isolated from various bloods by Davis, Newton, and Benedict [1922], or of other purine- or pyrimidine-carbohydrate compounds, would offer an explanation of the unassigned values in both the cold and hot methods, and is attractive in view of the peculiar position of uric acid in avian metabolism. Alternatively, the view may be taken that the unassigned cold and hot values have no relationship, and that the additional hot value represents either non-glucose carbohydrate or glucosephosphoric esters which have been formed during glycolysis or after the administration of insulin.

The non-glucose reducing substances of B-avitaminous pigeons' blood.

Analyses of the reducing substances in the blood of B-avitaminous pigeons have been made chiefly to determine whether the hot and cold reducing values were altered in these abnormal cases. The results are recorded in Table IX.

Table IX.

Results in mg. per 100 cc. blood.

	Zinc								
	Initial (calc. as glucose)		Glycolysed; time in brackets in hrs.		Trichloroacetic acid. Initial (calc. as glucose, neutralised)		Tungstic acid		
Bird	Cold	Hot	Cold	Hot	Cold	Hot	Total —SH (calc. as cysteine)	Indirect uric acid	Remarks
9	21	336	25 (11)	270	—	—	61	4·6	H.R.
10	34	425	29 (22)	288	—	—	—	—	H.R. *a*
11	—	216	—	—	32	255	—	—	—
12	21	195	—	—	21	207	—	—	H.R. *b*
13	—	115	— (6)	44	—	—	—	—	H.R.
14	18	368	—	48	—	—	—	—	H.R.
15	18	370	—	74	—	—	—	—	H.R.
16	26	287	—	—	26	285	—	—	H.R.

H.R. indicates head retracted. *a*, very abnormal bird. *b*, no symptoms after being on diet 72 days.

Examination of Table IX reveals that there is no very significant variation between undieted and avitaminous pigeons as regards the cold values for zinc or trichloroacetic acid filtrates, and that the hot values for these filtrates in the case of polyneuritic birds have the same inter-relationship as in normal cases. The content of total sulphydryl compounds and of uric acid would seem to be unaffected by the change in diet. The cases have been selected so that they include not only birds showing polyneuritic hyperglycaemia (Nos. 9, 10, 14, 15), but also those exhibiting normal values (Nos. 11, 12), and one having a very abnormally low blood-sugar (No. 13).

In those pigeons in which the glucose content is very high the glycolytic system may be either impaired or unable through inactivation to deal with the large amounts of glucose, since in several instances (see Nos. 9 and 10) attempts to determine the residual reduction by removing glucose by glycolysis were unavailing. Recourse was therefore made to the following procedure (see Nos. 14 and 15). The blood was centrifuged, the respective volumes of corpuscles and serum noted, and the corpuscles suspended in sufficient isotonic saline to make up the volume of the blood. The glucose was then eliminated by glycolysis from the saline suspension and from a known mixture of a small part of the serum and a larger fraction of the corpuscular suspension. Glycolysis was almost complete in the corpuscle-serum mixture after 2½ hours, as was confirmed by determinations after a further period of 1½ hours. From these results the residual reduction for the whole blood by the hot Hagedorn and Jensen method was calculated. It is clear that the residual reduction, due to non-glucose substances, is of the same order as in normal pigeons, but that it fluctuates more widely.

Isolation of ergothioneine hydrochloride from pigeons' blood.

Ten normal pigeons were stunned and guillotined, and in each case the blood (120–150 cc. in all) was shed into 60 cc. of boiling 0·01 N acetic acid. After being boiled for one minute to complete coagulation, the combined extracts were filtered and treated exactly as described by Benedict, Newton and Behre [1926] in the isolation of ergothioneine from pigs' blood. The residue (12 mg.) of crude ergothioneine hydrochloride obtained by evaporating the final acid solution to dryness in a vacuum desiccator was almost free from aliphatic disulphide compounds (Walker's test). It crystallised when rubbed with a drop of 0·5 N hydrochloric acid, and the crystals were pressed on filter paper and dried at 105°. The yield of pure material was 5 mg. This material melted at 207–208° alone or when mixed with a pure specimen of ergothioneine hydrochloride obtained from ergot, M.P. 207–208° (Tanret [1909], compare Newton, Benedict and Dakin [1927]), gave a very positive reaction by Hunter's test in which the odour of trimethylamine was much in evidence, and was free from aliphatic sulphydryl and disulphide compounds. In an attempt to obtain a further supply for analysis, the blood from ten pigeons was worked up in the same way, but the product in this case (10 mg. in all) contained a relatively greater proportion of aliphatic disulphides, detected by Walker's test, and the amount was inadequate for recrystallisation. Since there can be no question that the substance isolated was ergothioneine hydrochloride, we did not think it justifiable to sacrifice the large number of pigeons which would be necessary to enable an analysis to be made.

Discussion.

As a practical issue of the above work, we think that zinc filtrates made by the Hagedorn and Jensen method are the most reliable material for the estimation of glucose in avian blood, because they contain apparently a smaller number of non-glucose reducing substances than other types of filtrate. Somogyi's method of precipitation at room temperature is stated to be reliable for human blood, but on the one occasion on which we tried it we found that the filtrate gave a "cold" value of 16 mg. per 100 cc. (calculated as glucose) and a "hot" value of 299 mg. The figures obtained for the normal Hagedorn and Jensen filtrate of the same blood were 26 and 287 mg. respectively. Since Somogyi's filtrate presumably did not contain uric acid, glutathione, or ergothioneine [Somogyi, 1929, 1], it follows that it must have contained considerable amounts of other non-glucose substances. We regard the value of 16 mg. per 100 cc. obtained by the "cold" estimation of the Somogyi filtrate as additional evidence for the presence of the suggested unknown "cold" reducing substance.

Throughout this paper it has been assumed that absence of Hunter's ergothioneine reaction presupposes absence of thiolglyoxaline compounds, except in the case of trichloroacetic acid filtrates. It is possible, however, that ergothioneine may exist in the corpuscles in equilibrium with its —S.S— form, and that this oxidised form does not give the Hunter reaction. This compound has been obtained by Tanret [1909] and by Barger and Ewins [1911] as a periodide, but has not yet been isolated from blood. No information as to its behaviour towards the diazo-reagent is available, but experiments bearing on this point are in progress.

It is of considerable importance from the standpoint of comparative observations upon birds and mammals to know that the working concentrations of glucose in the tissues of the two classes are not widely different.

The results for the avitaminous birds settle a possible objection to the conclusion of Kinnersley and Peters [1929] that the increased lactic acid in such brains does not depend upon the sugar content of the blood. If the concentration of non-glucose reducing substances in an avitaminous bird had been much less than normal, then an apparently normal "sugar" value would actually mean a higher glucose content. Since this is not true, the conclusions are valid.

Summary.

1. The residual reducing value after glycolysis in pigeons' blood is approximately 70 mg. "glucose" per 100 cc. by the Hagedorn and Jensen method, representing substances mainly in the corpuscles. This agrees with the value reached in insulin convulsions.

2. It has been deduced that the glucose content in the blood of pigeons kept without food for 24 hours is 135 mg. ± 15 mg. per 100 cc., being not so

widely different from that of mammals as suggested by the total reducing value of approximately 200 mg.

3. Filtrates prepared by different methods contain different proportions of ergothioneine, uric acid and glutathione (aliphatic —SH compounds). These substances can be estimated in terms of glucose (100) by a "cold" Hagedorn and Jensen method, their values being: uric acid 53, glutathione 17 (hot 45), and ergothioneine 56.

4. Zinc filtrates made by the Hagedorn and Jensen method are the most reliable for estimating reducing substances in avian blood, but these contain in addition to glucose, ergothioneine and some other unknown substances reducing the ferricyanide reagent. Approximately 60 % of the residual value is not accounted for by ergothioneine.

5. Zinc filtrates do not contain aliphatic —S.S— and —SH compounds.

6. There are no significant variations from the normal in B-avitaminous birds.

7. Ergothioneine hydrochloride has been isolated from pigeons' blood.

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XV. HEAT-STABILITY OF THE (ANTI-DERMATITIS, "ANTI-PELLAGRA") WATER-SOLUBLE VITAMIN B_2.

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WHEN Goldberger and his colleagues [1926] discovered in yeast the water-soluble vitamin B_2, the nutritional factor which they named "P-P" and considered was involved in the prevention and cure of human pellagra, its separation from the antineuritic vitamin B_1 was achieved by heating the yeast in an autoclave for 2½ hours at 15 lbs. pressure. By this degree of heat the antineuritic vitamin was destroyed but the yeast thus heated contained a dietary factor necessary to prevent dermatitis and maintain health and growth in young rats.

In the efforts of subsequent workers [Chick and Roscoe, 1927, 1928, 1929; Williams and Waterman, 1927, 1928; Hogan and Hunter, 1928; Reader, 1929] to unravel the complex formerly known as "vitamin B," autoclaved yeast and autoclaved yeast extracts have frequently been employed as sources of vitamin B_2 apart from vitamin B_1, and the former is usually referred to as the "heat-stable" or "more heat-stable" constituent of the complex.

There is, however, little precise knowledge as to the degree to which vitamin B_2 is stable to heat, or of its sensitiveness to the action of acids and alkalis at high temperature. At the present time such information is especially important in view of the recently reported discovery of other thermolabile and thermostable constituents of the vitamin B complex, in addition to the well-established factors B_1 and B_2 [Hunt, 1928; Williams and Waterman, 1928; Reader, 1928, 1929; Peters, 1929]. These researches are discussed later in this paper.

While the following experiments were in progress, Williams, Waterman and Gurin [1929] published their investigation of the effect of reaction upon the destruction of vitamins B_1 and B_2 in brewer's yeast, when heated for 6 hours at 120° in an autoclave. They observed the growth of rats on diets in which the heated material was the sole source of vitamin B_1 or B_2 respectively. They concluded that much of the original vitamin B_2 survived the above degree of heating if the reaction were that natural to yeast (p_H about 4·5) and more if it were more acid (p_H 1·0–2·0), and further that some at least of the vitamin B_1 could survive this treatment if the reaction were acid. If the material heated were alkaline (p_H 8–14) both vitamins were destroyed.

As far as we are aware the only approximately quantitative data in the literature on the heat-stability of vitamin B_2 are contained in a previous paper of our own [1927], where a comparison was made between the content of vitamin B_1 and vitamin B_2 respectively, in dried brewer's yeast before and after heating for 5 hours in an autoclave at 120°. Of the unheated yeast 0·05 g. sufficed to cure the collapse due to lack of vitamin B_1 observed in young rats on an otherwise complete diet, while no alleviation of symptoms was observed with 0·2 g. of the heated material. When the diet contained vitamin B_1 as Peters's [1924] antineuritic yeast concentrate, 0·2 g. daily of the unheated, or 0·3–0·4 g. daily of the autoclaved, yeast sufficed to maintain growth and prevent dermatitis. That is to say, yeast after exposure to moist heat at 120° for 5 hours, contained about 50 % of the vitamin B_2, but no significant amount of the vitamin B_1, present in the original material.

The present paper contains the results of a more precise determination of the stability to heat of vitamin B_2 as contained in two different yeast products. Washed, pressed brewer's yeast was used in one series of experiments (A, Table I); "Yeast Fraction 5," an extract made from the former with dilute acetic acid, in a second series (B, Table II).

Series A (Table I). Fresh brewer's yeast was washed with cold tap-water and filtered four times; it was then pressed. A weighed sample (No. 1) was removed and dried to serve as control material and for determination of dry weight. Five weighed portions of the remainder were taken, each puddled to a thick cream with an equal weight of water, the p_H being about 5·0 (bromocresol green). One portion (No. 2) was heated in a steamer for 2 hours at 98–100° and another (No. 4) autoclaved at 123° for 5 hours. Of the remainder, portions 3 and 5 were made alkaline by addition of strong sodium hydroxide to a p_H about 9–10 (thymol blue) and heated respectively as portions 2 and 4 above. Portion 6 was acidified with concentrated hydrochloric acid to p_H about 3·0 (thymol blue and bromophenol blue) and autoclaved for 5 hours at 123°, during which process the acidity fell to a p_H of about 3·5. The alkaline material in portions 3 and 5 similarly became less alkaline during heating. After heating, all five portions were dried to a convenient consistency in a hot room under a fan, 3, 5 and 6 being previously acidified to a p_H of about 4–5. Determinations of the dry weight were made in each final product, and. in the subsequent tests, the doses were calculated in terms of the dry weight of yeast contained. The values for p_H given above were obtained colorimetrically with indicators and are approximate only.

The method of vitamin B_2 assay was that previously described [Chick and Roscoe, 1928; Aykroyd and Roscoe, 1929]. Young rats, 35–45 g. weight, immediately after weaning, were placed upon the basal diet free from B vitamins for 1–2 weeks, during which period growth was arrested. Afterwards, they received a suitable daily dose of the material to be assayed for vitamin B_2 and 0·1 cc. (the equivalent of 0·6 g. yeast) of Peters's [1924] antineuritic yeast concentrate, as source of vitamin B_1. The growth of the rat was then observed

Table I. *Heat-stability of vitamin B_2 in brewer's yeast, determined by observations on the growth of young rats receiving doses of the material before and after heating, respectively, as sole source of vitamin B_2 in a diet* complete in other respects and including vitamin B_1 as Peters's antineuritic yeast concentrate (daily dose equivalent to 0·6 g. original yeast).*

Exp.	Material (Yeast XVII)	Reaction during heating p_H approximate	Daily dose in terms of dry yeast g.	Litter	Rat	Body-weight at beginning of the test g.	Weekly increments of body-weight during the period of the test g.	Average (1st week excluded in expts. 1, 2, 4, 6)	Mean
1 Control	Washed, pressed, dried	—	0·25	1366	504 ♂	47	20, 19, 14, 13, 17	16	16
			0·2	1367	516 ♀	33	11, 14, 17, 11, 11	13	14
			0·2	1378	525 ♀	32	16, 17, 15, 6, 14	13	
			0·2	1385	536 ♂	40	14, 12, 18, 16	15·5	
			0·1	1367	515 ♂	35	7, 10, 12, 6, 12	10	10
			0·1	1378	524 ♀	35	9, 12, 9, 9, 11	10	
2	Steamed 2 hrs. 98–100°	5·0	0·2	1385	534 ♀	38	12, 17, 9, 12	13	15
			0·2	1385	535 ♂	41	16, 16, 18, 16	17	
3	,,	9–10	0·4	1374	509 ♀	47	3, 1, 5, 4, 12	5	—
			0·4	1367	512 ♂	41	1, −1	0	—
			0·2	1385	533 ♀	39	1, 0, −2, 2	0	—
4	Autoclaved 5 hrs. at 123°	5·0	0·8	1438	545 ♂	47	29, 23, 15, 13	17	17
			0·4	1367	519 ♂	36	16, 14, 9, 11, 11	11	12
			0·4	1374	510 [illegible]	46	26, 14, 13, 11, 11	12	
			0·2	1378	526 ♀	33	15, 18, 11, 7, 11	12	11
			0·2	1367	520 ♀	35	10, 11, 10, 8, 9	9·5	
5	,,	9–10	0·4	1374	508 ♀	56	1, −2, 3, 6, 0	2	
			0·4	1374	505 ♂	47	5, 0	2·5	—
			0·4 (+0·1 g. dry yeast)	,,	,,	(52)	(3, 6, 7)	(5)	—
			0·4	1374	507 ♀	36	1, −1	0	—
			0·4 (+0·1 g. dry yeast)	,,	,,	(36)	(8, 9, 8)	(8)	—
			0·4	1367	517 ♀	35	−1, −1	−1	—
			0·4 (+0·1 g. dry yeast)	,,	,,	(33)	(7, 4, 6)	(6)	—
6	,,	3·0–3·5	0·8	1438	540 ♂	44	26, 29, 28, 23	27	27
			0·4	1367	514 ♀	42	12, 18, 12, 8, 9	12	11
			0·4	1374	506 ♂	44	19, 17, 7, 7, 10	10	
			0·2	1378	527 ♀	31	11, 12, 6, 4, 13	9	10
			0·2	1367	518 ♀	34	9, 13, 12, 5, 13	11	

* Specially purified caseinogen 20, rice starch 60, cotton seed oil 15, salt mixture (McCollum and co-workers [1917] No. 185) 5 water 100; cooked 3 hours in a steamer to prevent occurrence of refection [Roscoe, 1927]; cod-liver oil (0·05–0·1 g. according t size) administered daily to each rat to provide vitamins A and D.

for a period of 4–5 weeks. The preliminary period with complete deprivation of B vitamins is inserted in order to make the rats eager to consume the doses when offered subsequently.

The results of Exp. A are set out in Table I, the aim of the separate trials being to compare the various products with the original material, as regards the size of dose needed to induce resumption of growth to an equal degree.

A rat which is limited in growth by absence from its diet for 1–2 weeks of vitamin B_2 and subsequently receives it, appears to be relatively insensitive to large variations in the amount administered, especially if the daily dose is near to that required for normal growth. As large a difference as 100 % in the dose given may be followed by a disproportionately small difference in the amount of growth. The quantitative conclusions drawn from the data in Tables I and II have, therefore, a wide margin of error.

The results in Table I may be summarised as follows. Yeast at its ordinary reaction (p_H about 5·0) suffers no determinable loss in vitamin B_2 content after being steamed for 2 hours at 98–100°. The average weekly increase in weight of 2 rats receiving doses of the heated material equivalent to 0·2 g. dried yeast was 15 g., while that of 3 rats receiving 0·2 g. of the original dried yeast was 14 g. After being autoclaved for 5 hours at 123° about half the original potency is destroyed; the average weekly increase in weight of rats receiving doses equivalent to 0·2 g. dried yeast of the autoclaved material and 0·1 g. of the original, being similar, viz. 11 g. and 10 g. respectively.

With alkalised yeast (p_H 9–10), however, whether steamed (Exp. 3) or autoclaved (Exp. 5), the destruction of vitamin B_2 appeared to be complete. With the doses given no significant growth was obtained. It was noticed, however, that the animals suffered severely from diarrhoea and it is probable that the total lack of growth was due, not only to deficiency of vitamin B_2, but also to the effect of toxic substances formed in the alkaline yeast during heating. In order to test this point 3 rats (505, 507, 517, Exp. 5), which for 2 weeks had shown no growth with doses of the autoclaved product equivalent to 0·4 g. dry yeast, received in addition 0·1 g. unheated dry yeast and were observed for a further period of 3 weeks. During this period the average weekly increase in weight was 6·4 g. as compared with 10 g. increase shown by animals which received the 0·1 g. dose of dry yeast alone (rats 515 and 524, Exp. 1). This discrepancy points to the presence of some actively deleterious substance in the alkaline-heated yeast. Our next effort, therefore, was to find some substance containing less material of a protein-like nature from which toxic products could be formed when autoclaved. An extract made from yeast with boiling dilute acetic acid was found suitable for the purpose.

Series B (Table II). The material used was the first extract obtained in the preparation of Peters's antineuritic yeast concentrate. Washed, pressed yeast was added gradually to boiling water containing 0·01 % acetic acid in the proportion of 1 kg. (200 g. dry weight) to 2 litres solution. The mixture was brought again to the boil, boiled for 5 minutes and filtered. The filtrate was concentrated on a water-bath to a volume of 400 cc., so that 2 cc. contained the equivalent of 1 g. dry yeast. The material was acidified to p_H about 3·0 for storage. Although still rich in vitamin B_2 the extract contained only one-fifth of the total solids of the original yeast. From the concentrated solution measured volumes were removed, the control sample remaining at p_H 3·3 and others being adjusted by addition of strong NaOH solution to p_H 8·3 and 9·9 respectively.

Treatment was as follows. Steaming for 2 hours at 98–100° at p_H 8·3 (Exp. 9) and autoclaving at 122–125° for 4 hours at p_H 8·3, 9·9 and 3·3 respectively (Exps. 10, 11 and 12, Table II). In each case the p_H before and after heating was determined by means of a hydrogen electrode and it was found that during heating the alkaline material became less alkaline (Table II, Column 3). After being heated the samples were adjusted to the original volume and those which were alkaline were acidified to p_H about 3·0 for

Table II. *Heat-stability of vitamin B_2 in dilute acetic acid* (0·01 %) *extract made from brewer's yeast XII. Method of assay as in Table I.*

Exp.	Material (Yeast extract XII)	Reaction p_H	Daily doses expressed as equivalent of original dry yeast g.	Litter	Rat	Body-weight at beginning of the test g.	Weekly increments of body-weight during the period of the test g.	Average	Mean
7 Control	Fraction 5	3·0	0·4	1438	542 ♂	47	18, 25, 18	20	18
			0·4	1443	560 ♀	42	20, 14, 14	16	
			0·2	1443	558 ♀	41	11, 11, 11	11	11
			0·2	1468	566 ♂	43	12, 10, 12, 12	11·5	
8	Kept at room temp. 10 days	9·9–9·5	0·4	1468	572 ♂	39	17, 10, 14, 20	15	15
			0·2	1468	573 ♀	43	8, 5, 11, 8	8	8
			0·2	1506	597 ♀	44	12, 4, 7, 7	7·5	
9	Steamed 2 hrs. at 98–100°	8·3–8·0	0·8	1468	570 ♀	41	19, 18, 14, 12	16	16
			0·4	1468	571 ♂	44	15, 13, 14	14	14
10	Autoclaved 4 hrs. 122°	8·3–7·1	0·8	1438	543	50	6, 3	4·5	5·5
			0·8	1442	548	40	8, 5	6·5	
11	Autoclaved 4 hrs. 124°–125°	9·9–8·7	0·8	1468	568 ♂	45	3, 9, 4, 10	6·5	—
			0·8 (+0·1 g. dry yeast XVII)	,,	,,	(71)	(22, 19)	(20·5)	—
			0·8	1468	569 ♀	41	3, −1, 1	1	—
			0·8 (+0·1 g. dry yeast XVII)	,,	,,	(44)	(18, 23)	(20·5)	—
12	Autoclaved 4 hrs. 123°–124°	3·3–3·0	0·8	1438	544 ♂	45	23, 26, 15	21	21
			0·4	1443	557 ♂	46	16, 11, 13	13	12
			0·4	1468	567 ♀	38	12, 7, 11, 14	11	

storage. Owing to the limited supply of the material fewer rats were used than in Series A and the growth was observed for a shorter period, viz. 2–4 weeks.

The loss in vitamin B_2 content on autoclaving the acidified extract for 4 hours was about 50 %; similar to that observed with the acidified yeast. Rats receiving doses of the original extract equivalent to 0·2 g. and of the autoclaved material equivalent to 0·4 g. yeast showed a similar average weekly increase in weight, viz. 11 g. and 12 g. respectively (Exps. 7 and 12).

The loss of vitamin B_2 on heating in alkaline solution was much greater. About one-half the original potency was destroyed by steaming at 98–100° at p_H 8·3–8·0 and more than three-fourths by autoclaving for 4 hours at 122° (p_H 8·3–7·1). Rats receiving daily doses of the autoclaved extract equivalent to 0·8 g. yeast showed an average increase in weight of only 5·5 g. weekly (Exp. 10), whereas 11 g. weekly was the average for those receiving the original material equivalent to 0·2 g. yeast.

After being autoclaved for 4 hours at 124° in more alkaline solution (p_H 9·9–8·7) one test indicated complete destruction of vitamin B_2, another over 75 % destruction (rats 568, 569, Exp. 11). When, later, these two rats received 0·1 g. dried yeast in addition to their dose of autoclaved extract, the response in growth was instant and complete, showing that the previous failure was not attributable to the effect of any toxic material produced in the alkaline yeast extract during heating (compare Exp. 11, Table II, with Exps. 5 and 1, Table I).

A specimen of the alkalised extract (p_H 9·9) was protected from access of CO_2 and left at room temperature for 10 days during warm weather

(September 6–16, 1929). At the end of the period the material was acidified and the vitamin B_2 content assayed. The solution appeared to have lost about one-third of its original potency, doses equivalent to 0·4 g. yeast caused a growth response intermediate between those evoked by doses of the original material equivalent to 0·4 and 0·2 g. yeast (Exp. 8, Table II).

The results of Exps. A and B may be summarised as follows. Vitamin B_2 is comparatively stable to prolonged heating at high temperatures if the reaction is acid, p_H 5·0–3·0, about 50 % of the original content surviving 4–5 hours' heating at 122–124°. If alkaline, p_H 8·0–10·0, however, although less labile than vitamin B_1, vitamin B_2 loses 30 % potency in 10 days at room temperature, 50 % on steaming for 2 hours and 75–100 % on heating for 4–5 hours at 122–125°.

DISCUSSION.

Since the discovery that "water-soluble B" contains at least two well-defined components, vitamins B_1 and B_2, evidence for the existence of an additional constituent has been brought forward from three different laboratories [Williams and Waterman, 1927, 1928; Hunt, 1928; Reader, 1929]. These researches have been summarised by Peters [1929].

One of these factors is stated to be thermostable and two thermolabile. All appear to be different from one another. None has been identified with prevention or cure of any specific disease or pathological syndrome. Their existence has been postulated to explain the failure of rats to grow to maturity (or of adult pigeons to maintain their weight) on diets which are believed to contain adequate supplies of the known necessary food factors, including vitamins B_1 and B_2. In some of these investigations vitamin B_2 has been supplied as strongly heated yeast or yeast products, the assumption being that the latter vitamin is entirely heat-stable.

Hunt [1928] has produced evidence showing that autoclaved yeast contains a heat-stable B vitamin necessary for rat nutrition in addition to vitamin B_2 and that this factor is contained in whole wheat in addition to vitamins B_1 and B_2.

Williams and Waterman [1927] found that the B vitamins required for normal growth of the rat could be supplied by an antineuritic concentrate prepared with fuller's earth from an aqueous yeast extract supplemented with autoclaved yeast. This combination could not, however, maintain weight in the pigeon. From the fact that this was accomplished by whole unheated yeast, the authors concluded that a third unknown B vitamin, unnecessary for growth of the rat, was required for nutrition of the pigeon and was heat-labile. Peters [1929] has confirmed this observation, using unheated marmite.

A recent paper by Reader [1929] announces the discovery of a "second thermolabile water-soluble vitamin necessary for the nutrition of the rat." The basal diet contained all the necessary food constituents except B vitamins and when these were provided, as 6 % marmite, normal development of young rats took place. Growth, however, was subnormal and after a few weeks failed altogether, when vitamin B_1 was provided by Peters's antineuritic concentrate and vitamin B_2 as 6 % alkaline (p_H 9) marmite heated for 1 hour at 120°. It is assumed that the latter material contained an adequate supply of vitamin B_2 and the growth failure is attributed to destruction of a third B vitamin, "vitamin B_3," which is present in the raw marmite and is heat-labile. In view of the demonstration in the present paper of the serious degree of destruction suffered by vitamin B_2 in yeast and yeast extracts when these are heated at an alkaline reaction, it cannot be accepted, in the absence of quantitative experiments, that adequate amounts of vitamin B_2 survived the heating to which the marmite was subjected in Reader's experiments. And it seems doubtful whether the effect interpreted as due to qualitative deficiency of a new vitamin, "B_3," might not have been caused by an insufficiency of vitamin B_2.

Reader suggests (p. 693) that traces of "vitamin B_3" are probably present in Peters's antineuritic concentrate as at first obtained after extraction of the norite with dilute acid alcohol, and that these are removed in the further purification by fractionation with alcohol [Kinnersley and Peters, 1927]. In her experiments, vitamin B_1 was supplied as the more purified concentrate; in ours the less pure was used. We have obtained normal growth of rats to maturity (6–7 months) when vitamin B_2 was provided as autoclaved (slightly acid) yeast and vitamin B_1 as the less purified Peters's concentrate (unpublished experiments). It might, therefore, be suggested that in these experiments the "heat-labile vitamin B_3" was contained in our preparation of vitamin B_1. The fact, however, that the same vitamin B_1 concentrate was unable to supplement our vitamin B_2 preparation, when the latter had been strongly heated in slightly alkaline solution, indicates that, notwithstanding Reader's statement to the contrary [1929, p. 690], vitamin B_2 is a "heat-labile factor," and it is unnecessary to postulate the existence of another.

It would be more satisfactory if in the assay of vitamin B_2 the criterion could be the prevention or cure of the dermatitis (? rat-pellagra) developed in rats deprived of this vitamin, rather than the maintenance of growth. The irregularity in the time of onset of symptoms and in the degree of their severity would, however, make such observations untrustworthy and quantitative results impossible. Nevertheless, tests of the curative value of the control and heated materials would be useful to confirm our conclusion that it is vitamin B_2 rather than a third heat-labile vitamin B_3 which is destroyed when yeast or its extracts are heated at high temperature at alkaline reaction.

SUMMARY.

1. The anti-dermatitis (? anti-pellagra) vitamin B_2 contained in brewer's yeast and in an extract made therefrom, was found to be much more stable at high temperatures in acid (p_H 5·0–3·0) solutions than when the reaction was alkaline.

2. A yeast extract made with boiling dilute acetic acid (0·01 %) was found to be more suitable material for the study than yeast itself, owing to the formation of toxic substances in the latter during heating, especially when the reaction was alkaline.

3. At p_H 5·0 no loss in vitamin B_2 potency could be detected on heating yeast for 2 hours at 90–100°; about 50 % was lost on heating the yeast for 4–5 hours at 123°; at p_H 3·0 the loss was the same both with yeast and yeast extract.

4. When the reaction was alkaline (p_H 10–9·5) about 30 % of the vitamin B_2 originally contained in the yeast extract was lost in 10 days at room temperature (summer); on heating for 2 hours at 98–100° (p_H 8·3) the loss was about 50 % and on autoclaving for 4–5 hours at 122–125° (p_H 8·3–10) between 75 % and 100 %.

5. These facts concerning the sensitiveness of vitamin B_2 to high temperatures in alkaline solution are of importance in connection with the recently reported discovery in yeast of a new thermolabile B vitamin, in addition to the antineuritic vitamin B_1, the assumption in one at least of these researches being that vitamin B_2 is heat-stable even in alkaline solution.

Our thanks are due to Miss A. M. Copping for her assistance in caring for the rats used in the experiments of Series B.

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XVI. SPHINGOMYELIN, PHRENOSIN AND KERASIN IN FILMS ONE MOLECULE THICK ON WATER.

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(Received January 7th, 1930.)

The cross section of the paraffin chain has been measured in crystals by the X-ray method and found by Müller [1927] to have the value 18·3 sq. Å. The area occupied in films one molecule thick formed upon water by compounds containing long chains of carbon atoms has been measured by Adam [1922, 1928] and has been proved to have as its minimum value about 20·5 sq. Å. He has described and defined conditions in which larger values than this are found for substances that, under other conditions, give this value, and has especially studied the influence exerted on the dimensions of the films by the character of the polar group at the end of the chain, by reason of which the formation of the film is made possible and the molecules are anchored to the water surface. Compounds with a large variety of such polar groups have been investigated and the foundations have been laid on which much that is of interest to biochemists must rest. Most of the substances of biochemical importance in which long fatty chains occur are not of a simple character; they contain commonly more than one such chain and their polar groups present great variety and complexity. Some of these Adam has dealt with, *e.g.* glycerides and lecithin. But many such substances present difficulties which must be approached with circumspection, and only after the ground has been thoroughly surveyed. Moreover they are in many cases difficult to obtain with sufficient guarantee of purity.

On two grounds however we felt justified in experimenting with some of the less familiar substances of biochemical interest even at this stage. Specimens were available that were probably as pure as any hitherto obtained; they were prepared by Dr Rosenheim and given by him to this department for work of this nature: and secondly, though their constitution has not in all particulars been finally settled, enough has been clearly established for them to present points of interest in a study of their behaviour in films on water.

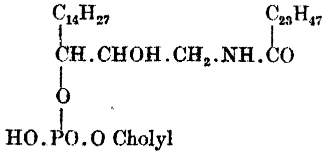
The substances dealt with in this paper resemble one another in two respects; they contain two long aliphatic chains similar if not identical in all three; these are a primary amine sphingosine and a peculiar long-chain fatty acid, and these two components are united as an acid amide $R_1.CO.NHR_2$.

The constitution of the base sphingosine, $C_{17}H_{35}O_2N$, is almost completely known. Levene and Jacobs [1912] showed that the whole of the nitrogen was removed by nitrous acid; that since a triacetyl derivative could be obtained the two oxygen atoms were present as alcoholic hydroxyl groups; and that a doubly linked pair of carbon atoms was present in the chain, since by means of palladium a dihydro-derivative could be formed. Lapworth [1913] on oxidation of sphingosine with chromic acid obtained normal tridecylic acid. Levene and West [1914] by oxidation of dihydrosphingosine obtained normal pentadecylic acid. This leaves no doubt that the double bond is between the 13th and 14th carbon atoms and that one hydroxyl group is attached to the 15th; there is little doubt that the other is attached to the 16th and the amino-group to the terminal or 17th carbon atom, though this has not been proved.

The fatty acid, lignoceric acid, $C_{24}H_{48}O_2$, in sphingomyelin is, according to evidence furnished by Levene [1916], united to sphingosine as in an acid amide, a derivative having been obtained containing base and acid, but not the other components of the molecule, and this derivative gave up no nitrogen to nitrous acid. The same union exists apparently also in phrenosin and kerasin [Levene and West, 1912], as neither of them contains a free amino-group. In kerasin therefore the remaining component, galactose, must be condensed with one of the hydroxyl groups of the sphingosine, as a galactoside, since the compound has no reducing properties; and in phrenosin too, though there is here another possibility, seeing that phrenosinic acid is a hydroxy-acid, the probability is that the same arrangement holds. Similarly in sphingomyelin one of the hydroxyl-groups of the sphingosine must be condensed with the cholylphosphoric acid.

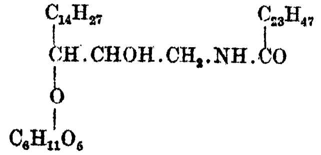
In all three compounds, therefore, the two long chains are connected to polar groups which are large enough to separate them much more than is the case with any of the compounds containing more than one long chain which have up to now been studied in this way, glycol esters, glycerides, lecithin or cetyl palmitate. Having regard to their power to form films one molecule thick on water they may be represented thus:

Sphingomyelin



$$\begin{array}{l} C_{14}H_{27} \qquad\qquad\qquad\quad C_{23}H_{47} \\ \mid \qquad\qquad\qquad\qquad\quad \mid \\ CH.CHOH.CH_2.NH.CO \\ \mid \\ O \\ \mid \\ HO.PO.O\ \text{Cholyl} \end{array}$$

Kerasin



$$\begin{array}{l} C_{14}H_{27} \qquad\qquad\qquad\quad C_{23}H_{47} \\ \mid \qquad\qquad\qquad\qquad\quad \mid \\ CH.CHOH.CH_2.NH.CO \\ \mid \\ O \\ \mid \\ C_6H_{11}O_6 \end{array}$$

In phrenosin the right-hand chain of this latter formula becomes $C_{24}H_{48}O$ according to Levene and Taylor [1928], $C_{23}H_{47}O$ according to Klenck [1928;

see also Taylor and Levene, 1929]. In all these compounds the two paraffin chains are at the two ends of the same chain with polar properties consisting of four carbon atoms and one nitrogen atom. Attached to this polar chain, in the case of sphingomyelin, is the additional polar group cholyl-phosphoric acid, in the case of kerasin and phrenosin the additional polar galactoside group. Whether these additional polar groups attached to the chain common to all three are attached as represented above or by the other alcohol group further to the right is not known; but for present purposes this is immaterial.

EXPERIMENTAL.

The apparatus used was that of Adam and Jessop [1926] and throughout this work the authors have had the benefit of Dr Adam's guidance in the use of it. All the solvents used were specially distilled, so that there was no contamination of the surface of the trough due to the presence in them of foreign matter. The solvent used was a mixture of pyridine and benzene. The sphingomyelin, weighed out accurately, was first dissolved in a little pyridine, which was warmed gently until solution was complete; then after cooling, benzene was added, the volume used being roughly 2–3 times the volume of the pyridine. It was found that the addition of a small amount of pure ethyl alcohol gave a clearer and more stable solution. The solutions were then weighed and were used on the trough immediately after they had been prepared, because it was found that after standing for any considerable period of time, especially in cold weather, part of the sphingomyelin often came out of solution. This behaviour was carefully watched for throughout the experiments, and as soon as it was observed the solution was rejected.

The films were studied at a number of temperatures between 0° and 35°. In general three or more readings were taken for each solution at each temperature. Although certain small deviations were observed between different solutions, these were never large and most of the curves obtained were practically coincident.

The curve for sphingomyelin, which is reproduced, represents the one which was most frequently obtained at room temperature and it forms a good average of the small experimental variations. At this temperature the area per molecule at the commencement of packing (*i.e.* the area of the head of the molecule) is about 60 sq. Å. As the pressure on the film is increased, the molecules pack closer until the area occupied by each molecule is about 42 sq. Å., the area, that is, that should be given by a film in which two parallel paraffin chains are packed closely together. Further compression decreases the area per molecule to 39 sq. Å. but, at the pressure necessary for this (42 dynes/cm., more than 150 atm.), the film shows signs of collapse. At room temperature the film is liquid at low and medium compressions but solidifies at high compressions. At 0° the film solidifies at lower pressures, but at 25° and above the film is liquid under all pressures.

At 0° the head of the molecule occupies a smaller area (50 sq. Å.) but as the pressure is increased the curve becomes coincident with that obtained at room temperatures. At 25° and 35° the films of sphingomyelin become increasingly difficult to compress, and under the highest pressures obtainable without collapse of the film (about 45 dynes/cm.), the limiting area of 42 sq. Å. is never reached.

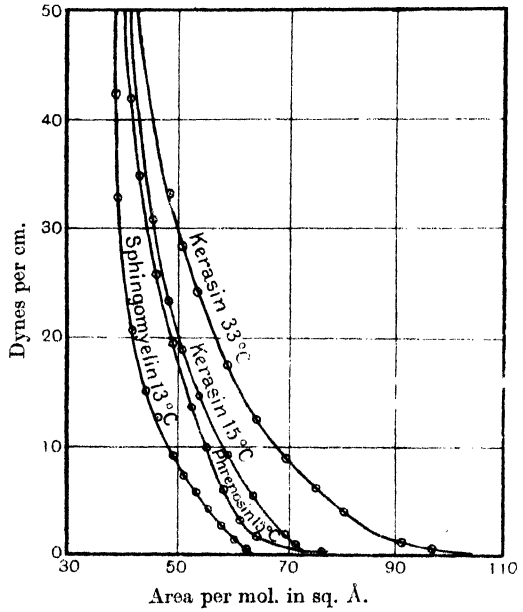


Fig. 1.

The curves obtained with phrenosin and kerasin were practically identical and closely resemble that given by sphingomyelin. The size of the head is about 70 sq. Å. at room temperature, and the limiting area per molecule under the highest compressions is about 42 sq. Å. At 33° the head occupies a larger area (about 100 sq. Å. per molecule) but as the pressure is raised, the curve gradually approaches that obtained at 15°. At room temperature the films are solid under moderate pressures, and at about 30° they are liquid under all compressions.

Discussion.

In the measurements here recorded it is to be noted that with sufficient compression the area occupied by molecules of these substances in films one molecule thick on water can be reduced nearly to that which their two paraffin chains alone would occupy when vertical to the surface and packed closely. The complexity of the polar groups connecting the two paraffin chains makes this remarkable. Compounds containing a single paraffin chain have been shown by Adam to resist compression to the area accounted for by that chain in varying degrees according to the nature of the polar group holding them to the water. Thus the areas of films of α-bromo-fatty acids, acetamides, ureas and monoglycerides are larger than those of saturated fatty acids, alcohols or esters. Monopalmitin and monomyristin for instance [Adam, Berry and Turner, 1928] at 11° and a linear compression force of 5 dynes/cm. occupy about 25·5 sq. Å.; as the compressing force is increased up to 30 dynes/cm. the area contracts uniformly to 23 sq. Å., altogether about 10 %. With our compounds containing two paraffin chains the area at 5 dynes/cm. is for sphingomyelin 54 sq. Å., or 27 for each chain and for kerasin 64, or 32 for each chain; at 30 dynes/cm. the area for sphingomyelin is reduced to 40, a contraction of 27 %, for kerasin to 46, a contraction of 28 %; so that with this substance the area per chain at this pressure is the same as in the case of the monoglyceride. The compression curve is however not rectilinear with either sphingomyelin or kerasin. With these substances therefore the area at low compression is definitely larger than for the monoglyceride, but they are more compressible than it is. It is remarkable that this should be so; not so much that at low pressure the completely immersible glyceryl group in $R.COO.CH_2.CHOH.CH_2OH$ should occupy less than half the area occupied by the complex polar group in these substances suspended between the two paraffin chains at its two ends, as that the latter should be more compressible. There seems to be more pliability in it than in the glyceryl ester group. The rigid bulkiness of this latter is not accounted for by the ester linkage; for with ethyl stearate the area of the molecule is reduced to that of the single paraffin chain at about 10 dynes/cm. [Adam, 1923, 2].

Measurements for compounds containing more paraffin chains than one exist only for the ester union of fatty acids to carbon atoms directly united to one another as in tripalmitin, glycol dipalmitate [Adam, 1922] and hydrolecithin [Leathes, 1923; Adam and Jessop, 1926, p. 368] of which the last is especially comparable with sphingomyelin. The unsaturated acid chains in lecithin make it non-comparable. At 5 dynes/cm. hydrolecithin occupies a smaller area, 47 sq. Å. or 23·5 for each fatty chain; at 20 dynes/cm. it contracts, 12 %, whereas sphingomyelin and kerasin contract 22 and 23 % respectively; but the area it occupies at this pressure is 41·4 sq. Å., which is not more than is accounted for by the fatty chains.

Other compounds containing more than one paraffin chain for which data have been published by Adam [1923, 1] are cetyl palmitate, which is insufficiently anchored to the water to give stable films, and pentaerythritol tetrapalmitate [Adam and Dyer, 1924], which at 5 dynes/cm. occupied 22·5 sq. Å. per fatty chain and at 20 dynes/cm. 20 sq. Å.

Measurements such as those here recorded are clearly insufficient to enable a precise conception to be formed of the disposition assumed by the polar groups immersed in the water. But some idea is shown of the amount of distortion that they undergo under compression and of the amount of force necessary to overcome the strains set up by this compression. The thickness of a condensed film of palmitic acid is 24 Å., and in that case 1 dyne/cm.

means a pressure of about 4 atmospheres. The thickness of the layer formed by the polar chains in these molecules buckling under pressure must be very much less than this and the pressure when the force is 30 dynes/cm. accordingly very much more than 120 atmospheres.

The fact that the films formed by these substances on water should be definitely liquid at room temperature, at any rate under the action of a moderate compression force, is worthy of note.

SUMMARY.

The surface films of sphingomyelin, phrenosin and kerasin have been studied. It is found that for each substance the area occupied by the molecule in a closely packed surface film can be compressed to about 42 sq. Å. This is in good agreement with what would be expected from the configurations of the molecules as deduced from their chemical behaviour, on the supposition that the limiting value is the area occupied by the paraffin chains set side by side vertical to the water.

We wish to thank Professor J. B. Leathes for his encouragement and advice during this work, which was suggested by him.

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XVII. THE PHOSPHORIC ESTERS OF ALCOHOLIC FERMENTATION.

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(*Received January 7th, 1930.*)

SINCE the isolation of a hexosemonophosphoric ester from the products of the fermentation of glucose and fructose by yeast juice the further investigation of these products has been directed towards obtaining more information on certain questions then briefly discussed [Robison, 1922], namely:

(*a*) the proportions in which the hexosemonophosphoric and hexosediphosphoric esters are formed during fermentation with yeast juice and the significance of the variations observed in different experiments:

(*b*) the suggested dual nature of hexosemonophosphoric ester and the chemical constitution of this ester, or its components, and that of hexosediphosphoric ester:

(*c*) the possible occurrence of other phosphoric esters among the products of fermentation.

The investigation was extended to include the products of fermentation of glucose and of fructose by yeast juice, dried yeast and zymin. The esters, freed from yeast proteins and excess of sugar, were separated into fractions according to the solubility of their barium salts, and the nature of these fractions was investigated, making use of micro-analytical methods to avoid the rapid wastage of material. This quantitative examination included the estimation of total and inorganic phosphorus, specific rotation, reducing power by the Hagedorn and Jensen method and by an iodimetric method, and "fructose" by Selivanoff's reaction. The further separation of the esters was carried out by fractional precipitation of the barium salts, crystallisation of brucine salts, and in other ways, the progress being followed at all stages by the aid of the above micro-estimations.

One result of this work, the isolation and identification of trehalosemonophosphoric ester has already been described [Robison and Morgan, 1928]. This ester was obtained from the fermentation of glucose and of fructose by dried yeast in presence of phosphates and was found in amounts representing up to 5 % of the total esterified phosphorus. The dried yeast used for the fermentations was shown to contain neither trehalosemonophosphoric ester nor trehalose in quantities sufficient to account for the amount of the ester found in the fermentation products, but the experimental data did not justify any conclusion as to the significance of this disaccharide phosphoric ester in the fermentation process. The ester, however, was shown to be readily fermented by dried yeast, yeast juice and zymin.

A more general account of these experiments is given in the present paper.

MATERIAL AND METHODS.

Yeast preparations.

(*a*) Yeast juice: prepared from English mild ale top yeast by the method of Buchner modified by Harden and Henley [1927].

(*b*) Dried yeast: prepared from English mild ale top yeast (in one experiment from a bottom yeast).

(*c*) Zymin: prepared from English mild ale top yeast.

Fermentation.

Method A. Glucose or fructose (30 g.) was added to 300 cc. yeast juice, or to 50 g. dried yeast or zymin mixed with 180 cc. water. A further 70 g. of the sugar was added in two or three portions during the fermentation. To the fermenting mixture, maintained at 28°-30°, a 20 % solution (0·56 *M*) of $Na_2HPO_4,12H_2O$ was added in six equal portions as indicated by the evolution of carbon dioxide in a guide experiment. Except when otherwise stated, the additions were made when the rate of evolution of gas had fallen nearly to the minimum (hexosephosphatase rate). The total amount of phosphate added was equivalent to about 7 g. P. Dried yeast or zymin was allowed to remain in the sugar solution for 1 hour before the first addition of phosphate.

Method B (used in the later experiments). 30 g. sugar was added to the yeast and, after the preliminary period, a solution containing both phosphate (20 %) and sugar (20 %) was allowed to run in continuously at a controlled rate throughout the fermentation. The fermentation was carried out in a large bottle immersed in a water-bath and connected by two rubber tubes with (*a*) a 100 cc. burette containing the phosphate-sugar solution, (*b*) a nitrometer, capacity 1 litre, in which the carbon dioxide evolved was collected over brine. At intervals of 5 minutes the bottle was vigorously shaken and from the volume of gas evolved the rate of addition of the phosphate sugar solution was regulated, in accordance with the plan of the particular experiment. The fermentation was stopped or portions of the fermenting mixture were removed at definite intervals of time after the last addition of phosphate.

Precipitation of proteins.

The precipitation of yeast proteins by heat coagulation, though employed in the first experiments which led to the isolation of fermentation hexosemonophosphate, was abandoned owing to the risk that a hexosemonophosphate might thereby be formed by partial hydrolysis of the hexosediphosphate in the solution. The method then adopted [Robison, 1922], namely precipitation of the protein along with the barium salts by alcohol, avoided this risk, but the successive extraction of the barium hexosephosphates from the mixture of salts and coagulated protein was laborious and difficult to carry out in a quantitative manner. Neuberg and Leibowitz [1927] have described a more rapid method for the preparation of hexosemonophosphate, in which the proteins are precipitated by heat coagulation after the solution has been neutralised to phenolphthalein. The protein-free filtrate, after the addition of calcium or barium acetate, is once more heated to boiling in a brine-bath and filtered hot, by which means the phosphate and nearly all the hexosediphosphate are removed as insoluble salts.

We have made some experiments to determine the extent to which hydrolysis of hexosediphosphate might occur under such conditions.

Exp. 1. Solutions of sodium hexosediphosphate (0·03 M), adjusted to different hydrogen ion concentrations, were heated for different periods of time at different temperatures. The degree of hydrolysis was measured by the increase in inorganic phosphate.

Exp. 2. 10 cc. of a saturated solution of barium hexosediphosphate, at p_H 8·4 ("neutral to phenolphthalein"), was slowly heated to 100° in a water-bath and maintained at the boiling point for a further 5 minutes. The inorganic phosphate liberated was then determined. The results are shown in Table I.

Table I.

			% P hydrolysed			
Exp.	p_H	Time of heating min.	70°	80°	90°	100°
1	6·9	30	0·8	2·4	6·5	16·0
1	7·8	30	1·0	2·6	8·7	18·0
1	8·4	5	0·2	0·6	2·1	5·5
2	8·4	15 min. up to 100° 5 min. at 100°	—	—	—	9·7

Since the phosphoric acid group in position 1 of hexosediphosphoric ester is much more readily hydrolysed than the remaining group, it is probable that in the above experiments a monophosphate would be formed in amount equivalent to the greater part of the inorganic phosphate set free. The use of high temperatures during the separation of the products must therefore result in some contamination of the fermentation monophosphate with the ester produced by partial hydrolysis of the diphosphate. For some purposes this might not be of serious importance, but it would be very undesirable in these experiments in which one of the main objects was to separate and identify the components of the monophosphoric ester formed during fermentation.

For the last six years trichloroacetic acid has been used in this laboratory for the precipitation of the yeast proteins and has been found satisfactory for this purpose. The acid is added to the fermentation mixture in amount equal to 4 % of the total volume; the mixture is thoroughly shaken and allowed to stand, preferably at 0°, for 2 hours before being filtered. The latter operation is much accelerated by the addition of a few drops of capryl alcohol. The possibility of the hydrolysis of hexosediphosphate occurring during this stage owing to the acidity of the solution (p_H about 4) was not overlooked, but repeated tests failed to indicate any increase in the amount of inorganic phosphate under experimental conditions.

General separation of the barium salts.

The acid protein-free filtrate was treated with barium acetate equal in weight to the crystalline sodium phosphate added during the fermentation, and was then neutralised to phenolphthalein with baryta. The greater part of the alkali was added in concentrated, hot solution in order to avoid unnecessary dilution, but the final adjustment to p_H 8·4 was made with cold saturated baryta solution. The precipitate was filtered off, washed with small quantities of water, pressed out as thoroughly as possible, washed with absolute alcohol and dried.

"10 % A" fraction[1]. The filtrate from the sparingly soluble salts was treated with one-tenth its volume of absolute alcohol and the precipitate filtered off, washed with a little 10 % alcohol, then with absolute alcohol and dried in a desiccator. In certain experiments no precipitate whatever was given at this stage. The reason for these anomalous results will be discussed later.

"Soluble A" fraction. The filtrate from the 10 % A precipitate was treated with basic lead acetate, avoiding any large excess of this reagent; the precipitate was decomposed with hydrogen sulphide, the lead sulphide being filtered off, washed with water and again treated with hydrogen sulphide. The combined filtrates and washings, after thorough aeration, were treated with baryta solution until pink to phenolphthalein and poured into twice the volume of alcohol. It is important that these precipitates should be thoroughly washed with absolute alcohol and dried rapidly in a vacuum desiccator.

"10 % B" and "soluble B" fractions. The soluble A fraction was thoroughly ground with ten times its weight of water, to which one-tenth the volume of alcohol was finally added before filtration. The insoluble portion formed the 10 % B fraction; on pouring the filtrate into twice its volume of alcohol the soluble B fraction was obtained.

Other fractions. The organic phosphate in the filtrate from the 10 % A fraction was not entirely precipitated by basic lead acetate, the amount remaining in solution representing about 1 % of the total phosphorus esterified during the fermentation. The greater part of this residual phosphorus was brought down by basic lead acetate and ammonia, but the barium salts recovered from this fraction ("ω") were highly contaminated with phosphorus-free compounds and could not readily be purified. The alcoholic filtrates from the soluble A and B fractions also contained small amounts of phosphoric esters which were not recovered.

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[1] These somewhat inaccurate terms used in the laboratory have been retained for convenience.

Micro-analytical methods.

Total and inorganic P were estimated by the modified Briggs method [Martland and Robison, 1926].

Reducing power was estimated by the Hagedorn and Jensen method, omitting the zinc hydroxide precipitation. Inconsistent results were at first obtained by this method when applied to the hexosephosphates. The reducing power, calculated as per cent. glucose, varied with the amount of substance used for the estimation and was markedly increased by traces of alkali. It was found that these differences could be nearly eliminated by adding 0·5 cc. of a 0·5N solution of sodium hydroxide to the solution before heating for 15 minutes in the water-bath. The reducing power, calculated from the glucose table, was increased by as much as 30 % and was nearly independent of the amount of substance taken. This modification was adopted in all subsequent estimations, the same amount of alkali being added to the blank. The value of the blank titration is not, however, altered by this addition nor is the reducing power of glucose itself increased.

Aldose was estimated by the iodimetric method [Macleod and Robison, 1929]. Caution was necessary in interpreting the values so obtained for the crude fractions, owing to the possibility that some of the contaminating substances derived from the yeast and precipitated with the phosphoric esters might react with iodine. It is probable, however, that errors due to this cause were usually small except in the case of the very impure ω fractions.

Selivanoff's reaction. A roughly quantitative method was employed for the estimation of the fructose equivalent by the Selivanoff reaction. A suitable quantity of the solution diluted to 5 cc. was mixed with 5 cc. of a 0·05 % solution of resorcinol in 5N HCl. A standard solution containing 0·2 mg. fructose was similarly treated. Both tubes were heated for 6 minutes in a boiling water-bath and, after cooling, the intensities of the colours were compared in a colorimeter. The results, though not very consistent, were useful in supplementing the aldose determinations, more especially on the purified fractions.

The values obtained for highly purified specimens of the four phosphoric esters by the above methods together with the specific rotations are shown in Table II.

Table II.

	P	Reducing power as glucose		"Fructose"	
		H. and J.	Iodine	Selivanoff	
	%	%	%	%	$[\alpha]_{5461}$
Barium hexosediphosphate	10·16	12	2	10	+ 3·5°
„ aldosemonophosphate[1]	7·85	36	41	0	+ 19·5°
„ fructosemonophosphate (Neuberg)	7·85	36	2	22	+ 0·7°
„ trehalosemonophosphate	5·57	0	0	0	+132°

Composition of the fractions.

Using these values and assuming the four esters to be the only compounds present having reducing properties or optical activity, the probable composition of the various fractions was deduced arithmetically. This assumption was not necessarily correct, but such an analysis might furnish evidence of the presence of other phosphoric esters. The application of this method in the early stages of the investigation, when only three esters were known, led to the isolation of the fourth, trehalosemonophosphate.

Table III.

				Percentage composition of fraction (barium salts)				
Experiment	Fraction	P in fraction as % of total P recovered	Hexose-diphosphate	Fructose-monophosphate	Aldose-monophosphate	Trehalose-monophosphate	Inorganic phosphate	P-free impurities
5	Sp. soluble	38·6	73	0	20	0	2	5
Yeast juice-fructose	10 % A	15·8	31	22	28	2	5	12
	10 % B	7·9	15	7	40	0	20	18
	Soluble B	37·3	6	16	68	1·5	1·5	7
6	Sp. soluble	18·6	89	1	3	0	3	4
Yeast juice-fructose	10 % A	18·3	43	11	28	0	4	14
	10 % B	16·6	28	6	39	1·5	11	14·5
	Soluble B	44·8	2	30	58	2·5	1	6·5
7	Sp. soluble	80·1	90	0	0	0	5	5
Zymin-fructose	10 % A	7·3	77	0	10	0	1	12
	10 % B	3·4	50	7	12	0	22	9
	Soluble B	7·5	10	12	50	14	0	14
13	Sp. soluble	85·3	72	0	0	0	20	8
Dried yeast-fructose	10 % A	No precipitate						
	10 % B	6·0	35	4	23	3	4	31
	Soluble B	8·4	10	11	50	17	0	12
15	Sp. soluble	85·1	91	0	0	0	0·5	8·5
Dried yeast-fructose	10 % A	4·3	81	0	4	2	3	10
	10 % B	2·9	20	3	16	20	3	38
	Soluble B	7·0	6	8	19	54	0	13
20 *b*	Sp. soluble	52·3	83	0	0	0	17	0
Dried yeast-fructose	10 % A	11·3	93	0	0	0	1·7	5·3
	10 % B	21·4	78	0	10	0	9·5	2·5
	Soluble B	14·3	14	8	37	28	0	13

For most fractions the values of reducing power, etc., calculated from the percentage composition so found, were in good agreement with those obtained experimentally, except that the Selivanoff and the iodimetric figures were in some cases mutually inconsistent. Both these values may have been affected by the impurities in the crude fractions and the figures showing the partition of the hexosemonophosphoric ester into aldose and fructose components are therefore of limited significance. This criticism does not apply in the case of the more highly purified fractions obtained in later stages of the separation. The percentage compositions deduced for the four chief fractions of experiments with yeast juice, dried yeast and zymin are shown in Table III.

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[1] Results obtained with the most highly purified specimen of the aldose component of hexosemonophosphoric ester. This component is probably glucosemonophosphate (theoretical iodimetric value 45·6). Evidence of the identity of the second component with Neuberg's fructosemonophosphate has been obtained [Robison and King, 1929, and further unpublished work].

The fact that the reducing power and other properties of these fractions could be quantitatively accounted for in this way does not prove that no other phosphoric esters were present, since mixtures containing one or more unknown esters might give equally satisfactory values. The evidence on this point will be considered in greater detail, taking each of the main fractions in turn.

Sparingly soluble fraction.

In the experiments with dried yeast and zymin, in which the three monophosphoric esters together represent less than 20 % of the organic phosphorus, the sparingly soluble fraction consisted almost wholly of hexosediphosphate together with most of the inorganic phosphate. A small amount of nucleic acid was present but was not separately estimated. The proportion of P-free impurities was usually small. In the yeast juice experiments, when the monophosphoric esters formed a much larger proportion of the total products, a certain amount of these soluble barium hexosemonophosphates was always carried down with the hexosediphosphate and in one experiment formed over 20 % of the fraction. This was confirmed by the separation of the hexosemonophosphate in nearly pure condition.

It was noticed that when the crude fraction contained much inorganic phosphate the whole of the diphosphate could not be extracted with 200 parts of water nor even by grinding the residue with further quantities of water. The amount of ester in the residue was roughly proportional to the inorganic phosphate and this suggested that an insoluble double salt had been precipitated. Further evidence in the same direction was found in the absence of any 10 % A precipitate in such experiments, indicating that the filtrate from the sparingly soluble fraction was unsaturated with respect to barium hexosediphosphate (see also experiments shown in Table IV).

10 % *A and* 10 % *B fractions.*

In dried yeast and zymin experiments the 10 % A precipitate also consisted very largely of hexosediphosphate, but the 10 % B fraction contained up to 30 % of the two hexosemonophosphates. In yeast juice experiments the percentage of hexosemonophosphate was high in both 10 % fractions, often exceeding that of the diphosphate. The occurrence of so large a proportion of the very soluble barium hexosemonophosphates in these fractions did not seem altogether reasonable and an alternative solution was considered, namely the presence of an ester having a sparingly soluble barium salt and intermediate reducing power.

The precipitates were ground with 10 parts of water and the filtered extract was treated with (*a*) one-tenth its volume of alcohol and, after filtration, (*b*) twice its volume of alcohol. The residue was again ground with water and brought into solution with the minimum amount of hydrochloric acid. The p_H of this solution was readjusted to 8·4 by the addition of baryta, and the precipitate filtered off. The filtrate was treated with alcohol as described above. These operations were repeated on the several fractions, the progress of the separation being followed by analyses. Certain of the fractions were then converted into brucine salts which were separated by fractional crystallisation from dilute alcohol and finally reconverted to the barium salts which were analysed. In this way the 10 % A and B precipitates were separated into hexosediphosphate and hexosemonophosphates of sufficient purity for identification, together with fractions of less certain nature. Provided that the latter were included in the computation, the amounts of mono- and diphosphate thus recovered agreed moderately well with those calculated from the analyses of the crude precipitates. The reducing power and other properties of these indefinite fractions were, however, not easy to reconcile with those of any probable mixture of the known phosphoric esters and suggested rather that they contained a new ester, possibly a disaccharide diphosphate having one free aldose group. In spite of much work on these fractions the evidence is still very conflicting, and no conclusion can yet be reached. Experience has shown that great caution is necessary before inferring the homogeneity of brucine salts of these phosphoric esters from their definite crystalline form.

Whatever may be the nature of these fractions, it is certain that the hexosemonophosphates are carried down along with inorganic phosphate and hexosediphosphate and may form a considerable percentage of the crude 10 % precipitates. Since pure barium hexosemonophosphate is not precipitated from its 10 % aqueous solution by the addition of one-tenth the volume of alcohol, its occurrence in the sparingly soluble and 10 % A and B precipitates is probably due to the formation of less soluble double salts with inorganic phosphate and hexosediphosphate. The following experiments support this view. Solutions of barium hexosediphosphate (saturated) and of barium hexosemonophosphate (2·5 %) were mixed with various quantities of dilute phosphoric acid (3·4 %) and were then made pink to phenolphthalein by the addition of baryta, the requisite amount of water being added before neutralisation to make the final volume of all the solutions equal. After filtration the amounts of total and organic phosphate remaining in solution were estimated and from these the amounts in the precipitate were calculated. In some experiments barium acetate (5 %) was added to the hexosephosphate solutions, in order to approach more closely to the conditions under which the 10 % A precipitate was formed. The results are shown in Table IV.

Table IV. *Precipitation of hexosephosphates from aqueous solution with inorganic phosphate.*

Phosphoric ester	Barium acetate %	P in solution before neutralisation mg. per 100 cc. Organic	Inorganic	P in precipitate after neutralisation mg. per 100 cc. Organic	$\frac{\text{Organic}}{\text{Inorganic}}$
Hexosediphosphate	0	60	12	22·7	1·90
	,,	,,	24	30·6	1·28
	,,	,,	36	35·9	1·00
	,,	,,	48	38·7	0·81
	,,	,,	60	42·1	0·70
Hexosediphosphate	5	18·8	12	8·0	0·67
	,,	,,	24	13·0	0·54
	,,	,,	36	15·5	0·43
	,,	,,	48	15·8	0·33
	,,	,,	60	16·1	0·27
Hexosemonophosphate	5	167	9·5	6·0	0·63
	,,	,,	28·5	14·0	0·49
	,,	,,	47·5	19·0	0·40

It is seen that although no alcohol was added to these solutions, very considerable proportions of the hexosephosphates were carried down with the barium phosphate, probably as double salts. The analogous double salt of hexosemonophosphate-hexosediphosphate is probably more soluble in water and is only precipitated in presence of high concentrations of the monophosphate or on addition of alcohol as in the 10 % precipitates.

Soluble B fractions.

The investigation of the soluble fractions from dried yeast and zymin experiments and the isolation of trehalosemonophosphate have already been described. The examination of the fractions from yeast juice experiments will be dealt with more particularly in a further communication, but certain points of interest may be mentioned. In order to satisfy the analytical values it was necessary to postulate the presence of small amounts of trehalosemonophosphate in these fractions, although this ester has not yet been isolated from yeast juice preparations, nor has any purified fraction been obtained with properties definitely indicative of its presence. On the other hand, certain fractions have shown the same general intermediate characters as those mentioned above, which were isolated from the 10 % precipitates. Whether these contain a hitherto unknown ester or represent merely residual mixed salts of hexosediphosphate, hexosemonophosphates and trehalosemonophosphate, as would be required by the analytical figures, remains to be proved. It is not improbable that the trehalose ester is formed during fermentations with yeast juice but that its presence is obscured and its isolation rendered difficult by the large proportion of the monophosphates. Similar but less extreme cases are shown by the zymin fermentations. The high rotation of the soluble B fractions ($[\alpha]_{5461} = +28^\circ, +30^\circ$) pointed to the presence of trehalosephosphoric ester to the extent of 14 and 16 % respectively. Isolation of the ester in pure condition proved very difficult and was only achieved in the case of one preparation. It is hoped that the isolation may be facilitated by means of the sparingly soluble crystalline barium salt described later in the paper.

The soluble B fractions of the dried yeast and zymin experiments contained by calculation an unexpectedly large proportion of barium hexosediphosphate, an average of 10 %. Unless the solubility of this salt is greatly increased in presence of the monophosphates, this may be a further indication of the occurrence of an unknown ester not included in the calculations.

ω fractions.

These very small fractions were so highly contaminated with phosphorus-free substances that no certain inference could be drawn with regard to the phosphoric esters except that the presence of trehalosephosphate was shown by the specific rotation whenever this ester formed a significant part of the fermentation products.

Table V.

						Percentage distribution of esterified phosphorus				
			Duration of fermentation (minutes)					Probable partition of hexosemonophosphate		
Exp.	Method of fermentation	Sugar	t_1	t_2	Phosphorus esterified %	Hexose-diphosphate	Hexose-monophosphate	Fructose-monophosphate	Aldose-monophosphate	Trehalose-monophosphate
Yeast juice:										
1	A	Glucose	155	40	97·3	67·0	32·5	2·5	30·0	0·5
2	,,	Fructose	155	40	97·7	47·5	52·2	13·0	39·2	0·3
3	,,	,,	160	45	97·6	41·5	58·0	17·0	41·0	0·5
4	,,	Glucose	155	40	99·0	61·2	38·1	13·6	24·5	0·7
5	,,	Fructose	140	25	99·5	39·3	61·0	12·0	49·0	0·7
6	,,	,,	145	25	98·8	35·0	64·0	19·0	45·0	1·0
ymin:										
7	A	Fructose	225	35	95·2	86·5	11·0	2·0	9·0	2·5
8	,,	Glucose	230	35	96·1	88·0	9·0	1·5	7·5	3·0
ried yeast *a*:										
9	A	Fructose	270	40	96·6	90·0	5·0	0·0	5·0	5·0
10	,,	,,	270	40	97·3	84·5	10·0	1·5	8·5	5·5
11	,,	Glucose	270	40	99·4	85·5	8·5	2·0	6·5	6·0
ried yeast *e*:										
12	A	Fructose	270	40	94·4	92·0	3·0	0·0	3·0	5·0
ried yeast *b*:										
13	A	Fructose	70	20	82·3	83·0	14·5	2·5	12·0	2·5
14	,,	,,	140	25	95·4	80·5	12·5	1·5	11·0	7·0
15	,,	,,	260	75	99·4	89·0	4·5	1·0	3·5	6·5
ried yeast *c*:										
16	A	Fructose	65	10	61·6	78·5	20·0	3·0	17·0	1·5
17	,,	,,	90	10	76·9	90·5	5·0	0·5	4·5	4·5
18	,,	,,	225	45	96·1	89·5	3·5	0·5	3·0	7·0
19 *a*	B	Fructose	110	5	89·0	82·5	9·0	1·5	7·5	8·5
19 *b*	,,	,,	110	305	96·0	80·0	8·0	1·5	6·5	12·0
20 *a*	,,	,,	80	5	80·0	79·0	15·5	3·0	12·5	5·5
20 *b*	,,	,,	80	35	94·0	81·0	13·5	2·0	11·5	5·5
ried yeast *d*:										
21 *a*	B	Fructose	80	5	89·0	66·6	30·4	8·5	21·9	3·0
21 *b*	,,	,,	80	19 hrs.	98·0	84·6	15·4	3·9	11·5	0·0

t_1 is the time interval between the first and last additions of phosphate.

t_2 is the time interval between the last addition of phosphate and the addition of trichloroacetic acid.

In the experiments with dried yeast and zymin, but not with yeast juice, there was a preliminary period of 1 to 1½ hrs. before e first addition of phosphate.

DISTRIBUTION OF PHOSPHORIC ESTERS IN THE PRODUCTS OF FERMENTATION.

From the foregoing discussion it is clear that the quantitative estimation of the phosphoric esters in fermentation products, by division into fractions according to the solubility of the barium salts, is likely to give erroneous results unless the composition of the fraction is further investigated by analysis. The proportion of the soluble hexosemonophosphates precipitated with the sparingly soluble hexosediphosphate and the amount of the latter remaining in the filtrate will vary according to the relative proportions of these esters and of inorganic phosphate in the fermentation products. Even the analytical methods described in this paper do not furnish entirely dependable results. Table V shows the distribution of the total esterified phosphorus based on the estimated composition of the fractions. The main division into hexosediphosphate, hexosemonophosphate and trehalosemonophosphate may be accepted as approximately correct, since other esters, if present, cannot form more than a very small fraction of the whole. The partition of the hexosemonophosphate into its aldose and fructose components is given with reserve as these figures are based, not on the theoretical iodimetric value for an aldosemonophosphate (45·6 %), but on the value (41 %) experimentally found for the purest specimen of this component yet obtained. Although there are grounds for believing this to be pure glucosemonophosphate, the disparity in the iodine reduction values is difficult to explain. In addition, both the Selivanoff and iodine values are liable to be affected by the impurities present in the crude fraction.

In Exps. 1 to 12 (method A, p. 120) each addition of sodium phosphate was made after the rate of evolution of carbon dioxide had fallen to the minimum and the fermentation was stopped at a similar interval after the last addition.

In the fermentations with yeast juice (1–6) the proportion of hexosemonophosphate was high and reached 64 % of the total esterified phosphorus in one experiment. Still higher percentages of hexosemonophosphate have been recorded by Neuberg and Leibowitz [1927] and by Harden and Henley [1929]. The presence of a very small proportion (1 % or less) of trehalosemonophosphate was deduced by calculation but this ester was not isolated from the products.

For the dried yeast fermentations five different preparations were used, *a*, *b*, *c*, *d*, being made from an English brewer's top yeast, while *e* was a bottom yeast. In Exps. 9–12 hexosediphosphate represented over 84 % of the organic phosphorus, while some 5 or 6 % was present as trehalosemonophosphate, this ester being isolated by the methods previously described.

The products of the zymin fermentations were similar to those obtained with dried yeast, but the proportion of trehalosephosphate was smaller. The ester was isolated from the products of Exp. 8.

Exps. 13, 14 and 15 (dried yeast *b*) were made in order to study the effect of varying the intervals between phosphate additions on the distribution of phosphoric esters in the fermentation products. The specific rotation of the three soluble B fractions, $[\alpha]_{5461} = +32°, +58°, +75°$, clearly indicated the very different proportions of trehalosemonophosphate to hexosemonophosphate. The percentage of the latter ester was highest (Exp. 13) when the fermentation was carried out rapidly and the concentration of inorganic phosphate was maintained at a high level. With longer intervals between phosphate additions (Exp. 14) the proportion of hexosemonophosphate decreased while that of trehalosemonophosphate increased. With still longer intervals (Exp. 15) hexosediphosphate increased, chiefly at the expense of hexosemonophosphate. In Exps. 16, 17 and 18 (dried yeast *c*) the variations were of the same general nature, having regard to the fact that a different yeast preparation was used and that the time intervals between phosphate additions were shorter than in the corresponding experiments of the previous series. In Exp. 16 the total period of fermentation was only 1¼ hours and no more than 61·6 % of the phosphorus was esterified, of which 20 % was in the form of hexosemonophosphate and only 1·5 % in the form of trehalosemonophosphate. Exps. 19, 20 and 21 were made by method B (p. 120). The phosphate-sugar solution was added continuously so that a high rate of evolution of carbon dioxide was maintained. In each experiment one half of the fermenting mixture was removed and treated with trichloroacetic acid 5 minutes after the phosphate addition was completed; the remaining half was allowed to ferment for further periods of 5, ½ and 19 hours respectively, further additions of sugar being made in Exps. 19 and 21. The results in each case show a decrease in the amount of hexosemonophosphate during the second period, but this is not so marked as in Exp. 15. In Exp. 19 *b*, 5 hours after the last phosphate addition, the production of trehalosemonophosphate reached the maximum value hitherto observed (12 %). In Exp. 21 *b*, and in other experiments not recorded, trehalosemonophosphate entirely disappeared during 19 hours' fermentation. The divergent results that may be obtained when otherwise similar experiments are carried out with different preparations of dried yeast are illustrated by Exps. 20 *a* and 21 *a*. This difficulty is intensified when the products of fermentation by yeast juice are investigated.

So far as this limited number of experiments can justify any general conclusion, it may be said that the production of hexosemonophosphate is maximum when inorganic phosphate is present in considerable excess and a high rate of fermentation is maintained; this hexosemonophosphate tends to decrease as trehalosemonophosphate accumulates when fermentation is continued with a low concentration of inorganic phosphate, but during very prolonged fermentation trehalosephosphate disappears while hexosediphosphate increases.

No significant differences have been observed between the results obtained with glucose and with fructose.

Similar conclusions with regard to the production of hexosemonophosphate have been stated by Neuberg and Leibowitz [1927], who used juice prepared from a bottom yeast. The recent work of Boyland [1929] on the sequence of the formation of phosphoric esters by dried yeast led him to conclude that trehalosemonophosphate is formed late in the reaction with phosphate.

Experiments shortly to be published (Harden and Robison) indicate that when the hexosemonophosphates are fermented by zymin a high rate of fermentation, comparable with the phosphate rate in the fermentation of fructose, may be produced. This, however, only occurs in the presence of inorganic phosphate and the phenomenon exactly resembles that which takes place when sugar is fermented in presence of phosphate. If excess of monophosphate be added, the rate eventually falls and a second addition of monophosphate does not cause any further increase.

PREPARATION OF THE PHOSPHORIC ESTERS.

For the preparation of the individual phosphoric esters it is important to choose a type of yeast and experimental conditions which will give a maximum yield of the desired ester. For the preparation of the hexosemonophosphates rapid fermentation with an active yeast juice would be chosen. The monophosphates can be isolated and purified by conversion of the soluble B fractions into brucine salts and recrystallisation from dilute alcohol and other solvents. For the preparation of hexosediphosphate and trehalosemonophosphate a moderately prolonged fermentation with dried yeast may be expected to give the best results. The diphosphate can be readily obtained in pure condition by extracting the sparingly soluble fractions with 200 parts of water at p_H 8·6. The clear filtrate, which should be free from inorganic phosphate, is treated with alcohol until a slight cloud forms and is then heated in 200 cc. portions to 70° in a water-bath and filtered hot. The diphosphate remaining in solution can be recovered in a somewhat less pure condition by adding more alcohol and again heating to 70°. Purification by means of the lead salt is of little advantage and often leads to hydrolysis during the decomposition by sulphuretted hydrogen. The inorganic phosphate always present in the 10 % B fraction must have arisen in this way. Barium hexosediphosphate should be dried at low temperatures. Hydrolysis to the extent of 20 % has been observed after heating *in vacuo* over phosphorus pentoxide at 100° for 6 hours.

A CRYSTALLINE FORM OF BARIUM TREHALOSEMONOPHOSPHATE.

Trehalosemonophosphate may be isolated from the soluble B fraction by means of its crystalline, sparingly soluble brucine salt. The final purification is much simplified by the aid of the crystalline barium salt which has been obtained during the last few months. The amorphous salt is very soluble in water, but if the solution is treated with alcohol (15–20 %) until the first signs of precipitation are observed and then left at room temperature, transparent needles separate out in the course of a few hours and continue to increase in quantity during some days. The concentration of alcohol may be gradually increased, but with 20 % alcohol almost the whole of the ester ultimately separates in crystalline form. The crystals are very sparingly soluble in cold water, but if brought into solution with the aid of heat or of dilute acid they can be reconverted into the amorphous soluble form by rapid precipitation with alcohol. They appear to contain water of crystallisation (5 mols), but otherwise to have the same composition as the amorphous salt. It is suggested that this crystalline form may consist of two or more molecules joined together through the barium atoms, that is of a double salt similar in type to those discussed earlier in this paper.

SUMMARY.

1. Methods are described for the separation and investigation of the phosphoric esters produced during fermentation of sugars by yeast preparations.

2. Sources of error in the conclusions drawn from results obtained with the aid of these and other methods are discussed.

3. The distribution of total esterified phosphorus between the four known esters, estimated by the application of these methods, is given for a number of fermentation experiments with yeast juice, zymin and dried yeast.

4. Some indication has been obtained of the presence of a fifth ester, but this can at most form only a very small proportion of the fermentation products. The evidence is not yet decisive.

5. The effect of varying the rate of addition of phosphate on the relative proportions of the different esters has been studied. Hexosemonophosphate was obtained in maximum yield when the fermentation was rapid and inorganic phosphate was in considerable excess. Trehalosemonophosphate, on the other hand, increased in amount when the fermentation was continued for some time after the addition of phosphate, but again decreased if the fermentation was prolonged.

6. A sparingly soluble crystalline form of barium trehalosemonophosphate is described.

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XVIII. THE COLOUR REACTIONS AND ABSORPTION SPECTRA OF STEROLS IN RELATION TO STRUCTURE.

By ISIDOR MORRIS HEILBRON AND FRANK STUART SPRING.

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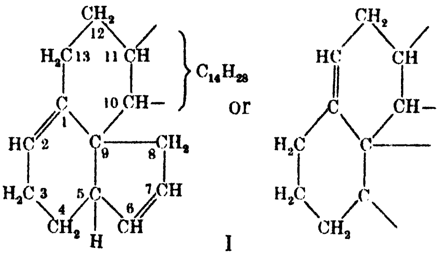
(*Received November 9th, 1929.*)

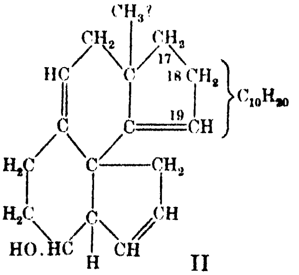
The production of an immediate carmine red colour with an aqueous solution of trichloroacetic acid has been shown, in all probability, to be dependent upon the presence of a $\Delta^{1:13}$ (or $\Delta^{1:2}$) ethenoid linkage in the sterol ring system [Rosenheim, 1929]. In accordance with this cholesterilene [Bose and Doran, 1929], *ψ*-cholestene, *allo*cholesterol [Windaus, 1927] and ergosterol [Heilbron and Sexton, 1929] all give the reaction, the latter being further characterised by the fact that the red coloration passes rapidly to blue.

Another reaction which has been stated to be specific for the detection of ergosterol or its derivatives (dehydroergosterol and *iso*ergosteryl acetate) is the green coloration produced by the Tortelli-Jaffé reaction [Häussler and Brauchli, 1929]. Employing this method the colour only develops slowly (about 10 minutes) and is only just discernible with 0·5 mg. ergosterol. The authors point out, however, that they have purposely not altered the standardised method of carrying out the reaction. We find that by employing the following modification the reaction can be rendered both instantaneous and more sensitive. A crystal of ergosterol is dissolved in glacial acetic acid (5 cc.) and 1 cc. of a 2 % solution of bromine in chloroform is introduced down the side of the tube by means of a pipette; a green ring appears at the surface of contact of the two solutions. Using these conditions, 0·02 mg. ergosterol can be immediately detected. The reaction is of peculiar importance, however, as the appearance of colour occurs, as shown in Table I, in hydrogenated derivatives of ergosterol which do not respond to either the Rosenheim or $SbCl_3$ reagent.

Whereas the compounds responding to the Rosenheim reaction are characterised by the presence of the $\Delta^{1:13}$ (or $\Delta^{1:2}$) double bond, it is evident that the appearance of colour in the bromine reaction is dependent upon some other ethenoid linkage. An insight into the position of this ethenoid linkage is gained in the first place from the non-reactivity of cholesterilene towards this reagent, and secondly from the positive reaction given by ergosterol. According to Bose and Doran [1929] cholesterilene may be represented as shown in formula I, whilst ergosterol is formulated as II [Heilbron and Sexton, 1929]. It follows from this that the bromine reaction is dependent upon the presence of the inert $\Delta^{10:19}$ (or $\Delta^{10:11}$) ethenoid linkage not present in cholesterilene. Thus both α- and β-dihydroergosterol to which the formulae III and IV have been respectively ascribed [Heilbron, Johnstone and Spring, 1929] fail to give the Rosenheim reaction, but react positively towards bromine. Again both α- and β-*iso*ergosterol (formula V) [Heilbron and Spring, 1929] show, as anticipated, both the Rosenheim and the bromine reaction. The same applies to dehydroergosterol ($C_{27}H_{40}O$, F_4); the presence of the "inert" linkage in this compound is demonstrated by the fact that hydrogenation leads to α-ergostenol [Windaus and Linsert, 1928].



I



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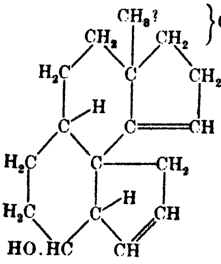

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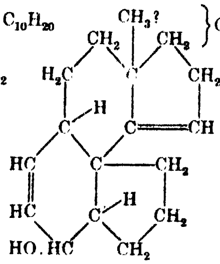
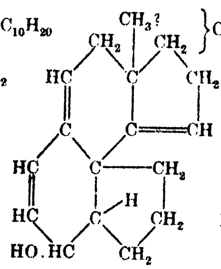
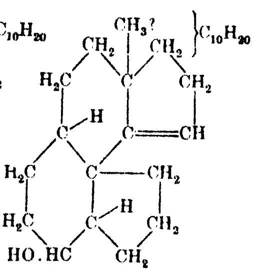


IV



V



VI

Table I.

Compound	Rosenheim reagent (trichloroacetic acid)	$SbCl_3$	Bromine	Absorption spectra
Ergosterol	Pink → blue	Red	Positive	Bands at 260, 270, 281, 293·5 μμ
α-Dihydroergosterol	Negative	Negative	Positive	No selective absorption
β-Dihydroergosterol	Negative	Negative	Positive	No selective absorption
α-*iso*Ergosterol	Pink	Yellow	Positive	Band at 247 μμ
β-*iso*Ergosterol	Pink	Yellow	Positive	Band at 247 μμ
Dihydro*iso*ergosterol	Pink	Yellow	Positive	Band at 247 μμ
Dehydroergosterol	Pink → green	Blue	Positive	Band at 313, 326, 344 μμ
α-Ergostenol	Negative	Negative	Positive	No selective absorption
β-Ergostenol	Negative	Negative	Negative	No selective absorption
allo-α-Ergostanol	Negative	Negative	Negative	No selective absorption
Cholesterol	Negative	Negative	Negative	No selective absorption
Cholesterilene	Carmine-red	Carmine	Negative	Bands at 294, 304, 321 μμ
Cholestene	Negative	Negative	Negative	No selective absorption
ψ-Cholestene	Carmine-red	Brown-red	Negative	No selective absorption
*allo*Cholesterol	Yellow-orange	Brown-red	Negative	No selective absorption
β-Cholesterol	Carmine-red	Red-brown	Negative	No selective absorption

The most important evidence regarding the specificity of the bromine reaction in relation to the "inert" ethenoid linkage is obtained from a comparison of α-ergostenol (tetrahydroergosterol) to which we ascribe the structure shown in formula VI, with its position isomer β-ergostenol [Reindel, Walter and Rauch, 1927]. Whereas the former compound fails to give, as anticipated, either the Rosenheim or the parallel antimony trichloride reaction, it readily shows the bromine coloration. On the other hand β-ergostenol gives no colour with any of the reagents. That the $\Delta^{10:19}$ double bond is absent from this compound is evident from the fact that it is readily hydrogenated to the fully saturated *allo*-α-ergostanol (hexahydroergosterol) which also fails to give colour reactions.

As shown in Table I no cholesterol derivative gives a positive reaction with bromine, a fact again explicable by the ease of complete hydrogenation of all such compounds and consequent absence of any "inert" linkage.

Selective absorption. Heilbron, Morton and Sexton [1928] have suggested that the power of selectively absorbing in the ultra-violet is only shown by sterol derivatives containing at least two double bonds in the molecule. It now appears that this statement can be further qualified as, of the sterol derivatives so far examined, only those show selective absorption which contain not only two double bonds but give positive colour reactions with trichloroacetic acid or antimony trichloride, *i.e.* which contain the $\Delta^{1:13}$ (or $\Delta^{1:2}$) ethenoid linkage. Thus ergosterol, α- and β-*iso*ergosterol, dihydro-*iso*ergosterol [Heilbron and Spring, 1929], dehydroergosterol and cholesterilene all give coloured solutions in presence of the Rosenheim reagent or $SbCl_3$, and all show well-defined bands in the ultra-violet. On the other hand although both α- and β-dihydroergosterol contain two double bonds in the molecule, the potent $\Delta^{1:13}$ linkage is absent and only general absorption is shown.

SUMMARY.

1. The Tortelli-Jaffé reaction has been modified so as to render it more sensitive. The reagent appears to be specific for sterol derivatives containing an "inert" (as applied to hydrogenation) linkage, possibly in position $\Delta^{10:19}$.

2. Selective absorption of sterols is conditioned by the presence of two ethenoid linkages in the molecule, one of which must apparently be in the $\Delta^{1:13}$ (or $\Delta^{1:2}$) position.

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XIX. ABSORPTION SPECTRA IN RELATION TO VITAMIN A.

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(*Received November 9th, 1929.*)

THE object of this paper is primarily to remove the confusion which appears to have arisen in a recent note on the absorption spectrum of vitamin A by Rosenheim and Webster [1929].

Takahashi *et al.* [1925], in work on "biosterin," recorded the fact that concentrates from the non-saponifiable fraction of liver oils exhibit selective absorption near 320$\mu\mu$ which was ascribed to vitamin A. Adverse criticism having made it clear that "biosterin" was very far from being pure vitamin A, it became uncertain whether the absorption band was due to the vitamin itself or to some other substance present in the concentrate.

Morton and Heilbron [1928] undertook to test the assumption that vitamin A exhibits selective absorption in the near ultra-violet, since the substance appears to undergo photochemical decomposition in sunlight. It was found that cod-liver oils, rich in the vitamin, exhibited a broad band in the region 300–350$\mu\mu$ (max. near 328$\mu\mu$), and that the material responsible for the band was concentrated in the non-saponifiable fraction. It was shown further that in all cases the intensity of the band was proportional to the intensity of the blue colour obtained with antimony trichloride. When the oils were irradiated by means of any light source emitting rays near 320$\mu\mu$, and when the oils were treated with oxygen, the absorption band near 328$\mu\mu$ and the capacity to give a blue colour disappeared at exactly the same rate. In all, the results for spectroscopic examinations both of blue colours and ultra-violet absorption for some 40 samples of fish oils, liver oils, concentrates and animal oils were observed without disclosing any departure from the strict parallelism between ultra-violet absorption, blue colour test, and, so far as was possible, growth-promoting action.

Drummond and Morton [1929] endeavoured to test the parallelism still more stringently. Six samples of cod-liver oil were examined, (*a*) fresh, (*b*) after storage for one year in darkness, (*c*) after storage for one year exposed to diffuse daylight. Throughout, the parallelism between the colorimetric and spectrographic tests was exceedingly close. Exhaustive biological tests on large numbers of rats were carried out on these oils, and considering the great difficulties in the way of a quantitative assay by animal tests, the agreement with the two physical tests was highly satisfactory. Further tests on sheep-liver fat and oil obtained from marine animals or fishes gave consistent results. More important perhaps from the present point of view was the fact that the richest concentrates of vitamin A obtained from liver fats gave a satisfactory growth response at precisely the dosage predicted from the intensity of the ultra-violet band and the blue colour. There is, therefore, a not inconsiderable body of evidence for stating that the growth-promoting factor present in liver fats exhibits selective absorption near $328\mu\mu$ and possesses the power of giving a blue colour (which has been defined spectroscopically) with antimony trichloride.

It will be observed that the claims have not been extended outside their proper sphere. The growth-promoting factor present in substances of vegetable origin may or may not be identical with the material in liver fats; there is no satisfactory evidence available yet. It is not claimed that if a substance promotes growth it must necessarily give the precise colour reaction of cod-liver oil and the near ultra-violet absorption band. To do so would be to make the unwarranted assumption that the vitamin A of liver fats is unique in the sense that it alone can produce a complex physiological response. It is, moreover, not claimed that if a substance absorbs near 300–$350\mu\mu$ it must be vitamin A. If such a claim were pushed to a logical, if extreme, conclusion, such diverse substances as citral, benzaldehyde and xanthophyll would have to be reckoned with.

The work on the vitamin A of liver fats rests essentially on an analytical basis. The best way to dispose of either the blue colour test or the ultra-violet absorption band as properties of the vitamin would be to concentrate the vitamin still further than has yet been done and to show that the parallelism to which reference has already been made ceases to hold.

Rosenheim and Webster [1929] consider that they have subjected the claim that vitamin A has a band near $328\mu\mu$ to the experimental test by preparing dehydroergosterol, and (*a*) confirming the absorption curve previously obtained by Windaus and Linsert [1928], (*b*) observing that the material does not promote growth, nor (*c*) give a blue colour with antimony trichloride. It is stated that dehydroergosterol possesses intense absorption exactly in the same region as that claimed for vitamin A, and "that selective ultra-violet absorption at 320–$328\mu\mu$, at any rate by itself, cannot be taken as a criterion of vitamin A."

The criticism advanced by Rosenheim and Webster means that if a substance such as dehydroergosterol exhibits selective absorption similar to that found in liver oils and concentrates, then such absorption cannot be ascribed to vitamin A. Such criticism is only valid if it be shown quantitatively that the selective absorption of liver oils in the near ultra-violet is actually due to dehydroergosterol. Otherwise, fortuitous coincidences cannot be ruled out. Whether the resemblance is indeed fortuitous is another matter; no relevant evidence has yet been brought forward.

In choosing dehydroergosterol as the reference substance it is conceivable that Rosenheim and Webster were acting on the suspicion that, since vitamin D is an ergosterol derivative, vitamin A might also be related to ergosterol. Accepting the idea as attractive if as yet but vaguely supported, let us submit that an argument valid for vitamin D might also be valid for vitamin A. Vitamin D and ergosterol appear to absorb selectively in roughly the same region of the spectrum. If, however, the criticism of Rosenheim and Webster with regard to vitamin A has any meaning, selective absorption at 260–300$\mu\mu$ is not a test for vitamin D. Admittedly such an incomplete description of a physical measurement would include many compounds. The chance of coincidence is, however, greatly reduced when the intensity of absorption, say a 0·01 % solution and a 1 mm. layer, is specified.

Now in comparing the absorption curve for dehydroergosterol and vitamin A, Rosenheim and Webster neglect the intensity factor. We have conducted a very careful re-investigation of dehydroergosterol [cf. Windaus and Linsert, 1928] and find that $\log I_0/I = 1{\cdot}0$ (*ca.*) with a 2 mm. layer of 0·02 % solution. A sheep-liver fat concentrate containing at most 10 % of vitamin A (according to estimates supplied by Prof. Drummond) absorbed to roughly the same extent with a 3 mm. layer of a 0·02 % solution. Hence if the band shown by the concentrate is due to vitamin A, the absorption of dehydroergosterol must be of a lower order. Not only is this the case, but the general shapes of the respective curves for vitamin A and dehydroergosterol are entirely different. Whilst the curve we have attributed to vitamin A has not so far been resolved[1], that of dehydroergosterol shows three distinct bands

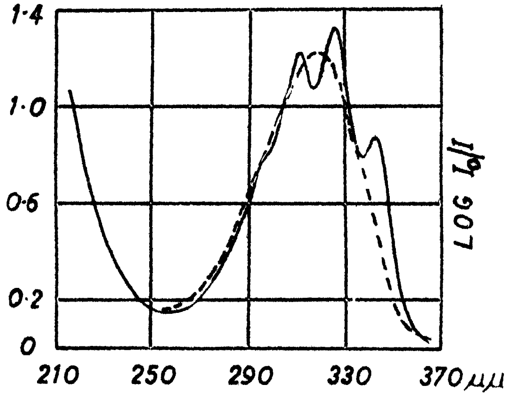

Fig. 1.

—— Absorption curve for dehydroergosterol; concentration 0·02 %; cell thickness 2 mm.

– – – The same selective absorption as observed without resolution (cf. Windaus and Linsert).

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[1] Evidence of ill-defined resolution is now being obtained using very rich concentrates. (*Note added February 3rd, 1930.*)

at 342, 326 and 311 $\mu\mu$ and an inflexion at about 297 $\mu\mu$ (Fig. 1). In fact the only proved resemblance is that the two curves extend between roughly the same limits. In view of this startling discrepancy it will perhaps be worth while to append evidence that our material is indeed dehydroergosterol.

The dehydroergosterol was prepared by boiling an alcoholic solution of ergosterol with mercuric acetate in alcoholic solution acidified with acetic acid, under the exact conditions described by Windaus and Linsert [1928]. After removal of mercurous acetate, the product was repeatedly crystallised from chloroform-methyl alcohol and finally obtained in large plates melting at 146°. The melting-point of dehydroergosterol is, however, in itself no trustworthy criterion of purity, as impure samples containing unchanged ergosterol melt at the same temperature as the pure product. We have consequently followed the purification throughout by means of the polarimeter. The portion used for the spectrographic examination had the following constant rotation:

$$[\alpha]_D^{19^\circ} = +149^\circ{\cdot}3 \ (c = 1{\cdot}59 \text{ in chloroform}).$$

Windaus and Linsert [1928] give

$$[\alpha]_D^{18^\circ} = +149^\circ{\cdot}2 \ (c = 1{\cdot}21 \text{ in chloroform}).$$

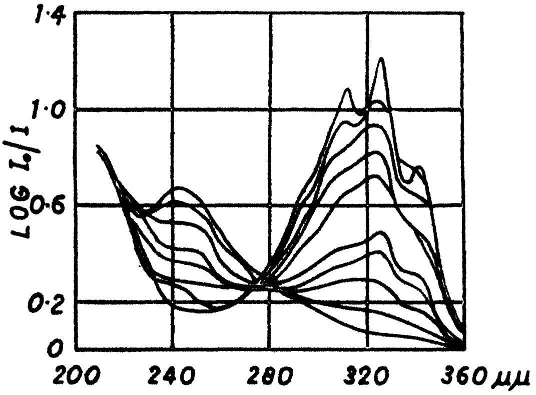

Fig. 2. Curves illustrating the disappearance of the characteristic absorption of dehydro ergosterol as a result of increasing periods of exposure to ultra-violet light. Initial concentration 0·02 %; cell thickness 2 mm. throughout.

In order further to characterise our product, dehydroergosterol was acetylated by boiling with acetic anhydride (10 parts) for 30 minutes. The material obtained after recrystallisation from absolute alcohol had physical properties identical with those described by Windaus and Linsert for dehydroergosteryl acetate.

Found: M.P. 146°; $[\alpha]_D^{19^\circ} = +193^\circ{\cdot}0 \ (c = 1{\cdot}5 \text{ in chloroform})$.

Windaus and Linsert give

M.P. 146°; $[\alpha]_D^{18^\circ} = +192^\circ{\cdot}5 \ (c = 2{\cdot}0 \text{ in chloroform})$.

It is not difficult to realise how the finer structure of the dehydroergosterol curve could escape detection by Windaus and Linsert if a photoelectric *method were used. By this method the intensity of absorption is determined* for selected wave-lengths of monochromatic radiation, *e.g.* 253–6, 265, 289, 303, 313, 336 $\mu\mu$, intermediate wave-lengths being neglected. With a photographic method, in conjunction with a perfectly continuous spectrum, the detailed structure of the absorption curve becomes apparent.

The photochemical decomposition of dehydroergosterol is a matter of some interest, and in Fig. 2 the result of exposure to the full radiation from a quartz mercury lamp using quartz test-tubes is shown. Among the products of the reaction is a substance exhibiting a broad band with a maximum near 247 $\mu\mu$. In the present preliminary work a current of nitrogen was kept bubbling through a 0·08 % solution in alcohol. The photochemical changes are being further studied under different conditions.

SUMMARY.

1. The criticism of Rosenheim and Webster [1929] that the selective absorption in the ultra-violet (broad band, max. 328 $\mu\mu$) of liver oils, in itself, cannot be taken as a criterion of vitamin A, is contested on general grounds.

2. The statement that dehydroergosterol exhibits an absorption band in the ultra-violet similar to that of vitamin A of liver oils is also contested, since this substance exhibits four narrow bands. The only similarity between the ultra-violet absorption spectra of dehydroergosterol and vitamin A is that they absorb in approximately the same region. Further, the intensity of absorption of dehydroergosterol is not greater than one-sixth of that shown by the absorbing constituent of liver oils.

In conclusion we desire to express our appreciation of the support given by the Department of Scientific and Industrial Research.

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XX. THE INHIBITION OF LACTIC ACID FORMATION IN CANCER AND MUSCLE.

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(Received December 5th, 1929.)

From a chemical standpoint, the problem of cancerous growth may be considered in two ways, either that disordered growth is caused by the presence of a chemical factor not present in normal tissue, or that disordered growth is caused by the absence of some factor normally present in adult tissue. The latter point of view seems, perhaps, to be the more plausible when one considers that, in certain respects, the metabolism of embryonic tissue and that of cancer tissue are similar [Warburg, 1924]. It seems reasonable to assume that with the development of the embryo there also develops a factor which checks certain phases of embryonic metabolism. This factor, present in normal adult tissue, would act as a growth regulator. If, in later life, the factor for any reason disappears, the tissue would again assume certain embryonic characteristics. In this paper experiments are described which were carried out to determine whether the addition of normal tissue extracts to cancer could change the characteristic metabolism of malignant tissue. As is well known, the work of Warburg [1923–1926] has shown that the most characteristic of the differences so far observed between the metabolism of cancerous tissue and that of normal tissue is the marked glycolytic power of the former. The line of experimentation followed, then, was to observe the change in glycolytic activity of cancer tissue brought about by the addition of extracts of various normal tissues.

Different regions of the body seem to possess a markedly different susceptibility to cancerous growths. The small intestine apparently affords a site very unfavourable to the growth of malignant tissue, since cancer in that region is extremely rare. On the line of thought suggested above, it might be supposed that the small intestine contained some chemical factor which checked cancerous growth, a factor either produced by the intestine itself or by one of those organs which empty their secretions into the small intestine. The pancreas thus suggested itself as the first tissue to use for our experimental purpose. A further reason for this choice was afforded by the observation of Winfield and Hopkins [1915], and later of Foster and Woodrow [1924] and Foster [1925], that extracts of pancreas had an inhibiting effect on lactic acid formation by muscle hash. McCullagh [1928] showed that pancreatic extracts also inhibit lactic acid formation in the soluble muscle enzyme system of Meyerhof [1926]. The present paper shows the effect of pancreatic extracts on cancer metabolism. This work was begun in May, 1928, and many of the experiments to be described had been completed when in December, 1928, a paper appeared by Barr, Ronzoni and Glaser [1928] in which the authors described experiments which had been undertaken with the same aim as our own, namely, to see whether pancreatic preparations inhibited the glycolysis of cancer tissue. Taking their results as a whole, they concluded that pancreatic extract did not inhibit this glycolysis. Our own experiments had led us to the opposite conclusion, and since some inhibition had been obtained in a number of Barr, Ronzoni and Glaser's experiments, we decided to continue our work.

EXPERIMENTAL.

Warburg has shown that not only is the anaerobic glycolysis of cancer tissue high, but also the aerobic glycolysis, whereas normal adult tissue shows practically no aerobic glycolysis. We therefore studied first the effect of pancreatic extracts on the aerobic glycolysis of cancer tissue. The type of manometric apparatus and technique described by Warburg were used. In all of these experiments, a thin slice of cancer tissue was suspended in bicarbonate Ringer solution (0·25M with respect to bicarbonate). When glycolysis was being measured, the Ringer contained 0·2 % glucose. Each apparatus was filled with a mixture of 95 % air and 5 % CO_2 and shaken in a water-bath at 37° for 1 hour. At the end of the experiment, the tissue was removed, washed, dried and weighed. A watery extract of commercial pancreatin was made by allowing 0·5 g. of the dry preparation to stand in 5 cc. water for an hour and then filtering. This preparation was made fresh every day. Another extract was made according to the method of Foster and Woodrow [1924] in which the factor is precipitated by 70 % alcohol. In all the later experiments, the pancreatic preparation from fresh pancreas described by Ronzoni, Glaser and Barr [1928] was used. This is precipitated by 58 % alcohol. Preliminary experiments showed that heated pancreatic extract did not inhibit the glycolysis of cancer tissue. In investigating the action of the pancreatic extracts, the same amount of heated extract was always added to the control apparatus as of unheated extract to the experimental apparatus, the two solutions being in this way more comparable than if water were used in the control. The pancreatic extract for the control was immersed in a test-tube in boiling water for 5 minutes. The p_H of heated and unheated extracts was brought to 7·6 before using.

The malignant tissue used was for the most part mouse carcinoma 63, and occasionally the slow growing Twort carcinoma, both kindly supplied by Dr J. A. Murray, F.R.S., of the Imperial Cancer Research Institute. Rat carcinoma 9 was tried but great difficulty was experienced in getting tumours that were not necrotic or haemorrhagic.

A typical experiment using mouse carcinoma 63 is given below. Five Barcroft micro-apparatus, of the type having the manometer open at one end, were used, containing:

1. 0·4 cc. bicarbonate Ringer + 0·1 cc. pancreatic extract + 2·8 mg. tissue:
2. 0·4 cc. bicarbonate Ringer + 0·1 cc. heated pancreatic extract + 2·2 mg. tissue:
3. 0·4 cc. bicarbonate Ringer (0·2 % glucose) + 0·1 cc. pancreatic extract + 1·6 mg. tissue:
4. 0·4 cc. bicarbonate Ringer (0·2 % glucose) + 0·1 cc. heated pancreatic extract + 1·8 mg. tissue.

A fifth apparatus containing 0·5 cc. Ringer only was used as a temperature control. Apparatus 1 and 2 contained no glucose and therefore gave the CO_2 produced by oxidation processes. Apparatus 3 and 4 contained glucose, and any CO_2 given off beyond that produced in 1 and 2 was due to the glycolysis of glucose [Warburg, 1923]. The figures (multiplied by the apparatus constant and divided by tissue weight) for the above experiment are given below. They represent $mm.^3$ CO_2 per mg. dry weight of tissue per hour.

1.	Ringer + pancreatic extract	+ 0·5
2.	,, + heated extract	− 1·7
3.	,, + glucose + pancreatic extract	+ 14·6
4.	,, + glucose + heated extract	+ 20·2

Glycolysis with pancreatic factor = (14·6 − 0·5) = 14·1
,, without ,, ,, = (20·2 + 1·7) = 21·9

In this experiment the pancreatic extract has caused an inhibition in aerobic glycolysis of 35 %. Previous workers using muscle hash had obtained inhibitions ranging from 40 to 60 %.

A possible source of error lay in the fact that the buffering power of the pancreatic extract might have been lowered by heating and that hence the heated extract would not retain so much CO_2 as the unheated. If this were true, a correction factor would have to be applied to the observed readings. To test this possibility, an experiment was carried out according to Negelein's [1925] method, and it was found that no extra retention of CO_2 occurred in the solution containing unheated pancreatic extract and that therefore no retention correction was necessary.

Table I gives the results of experiments measuring the aerobic glycolysis of cancer tissue as affected by extracts of pancreas. The pancreatic extract made according to Foster and Woodrow is designated as F and W. The extracts marked "sterilised" were passed through a sterilising filter.

It will be seen from Table I that in 15 of the 22 experiments the pancreatic extract caused an inhibition of glycolysis ranging from 15 to 100 %. During the course of these experiments, the work of Case and McCullagh [1928] appeared, which showed that the pancreatic factor inhibiting muscle glycolysis

Table I.

No. of exp.	Cancer tissue	Pancreatic extract	cc.	mm.³ CO_2 per mg. per hour			Inhibition %
				Without glucose	With glucose	Aerobic glycolysis	
1	Twort	Water extract	0·01 heated	− 1·35	+15·18	16·5	
			0·01	+ 1·4	+ 6·9	5·5	66
2	63	Water extract	0·1 heated	− 0·6	+ 8·6	9·2	
			0·1	+ 7·8	+12·0	4·4	52
3	63	Water extract	0·1 heated	+ 1·4	+19·2	18·8	
			0·1	+20·1	+30·8	12·7	32
4	63	Water extract	0·1 heated	+ 0·5	+15·7	15·2	
			0·1	+ 8·1	+ 8·1	0·0	100
5	63	Water extract	0·05 heated	+ 0·2	+19·1	18·9	
			0·05	+ 6·0	+21·9	15·9	16
6	63	F and W	0·1 heated	− 2·9	+ 4·9	7·8	
			0·1	+ 2·8	+ 4·7	1·9	75
7	63	F and W	0·1 heated	+ 3·5	+16·9	13·4	
			0·1	+ 6·7	+ 8·0	1·3	90
8	Spontaneous mouse tumour	Water extract	0·05 heated	− 0·5	+21·6	22·1	
			0·05	− 1·0	+16·9	17·9	19
9	63	Water extract sterilised	0·05 heated	− 2·8	+10·9	13·7	
			0·05	+ 2·4	+11·1	8·7	36
10	Twort	Water extract	0·05 heated	− 3·6	+11·6	15·2	
			0·05	+33·0	+27·4	—	100
11	63	Water extract	0·05 heated	− 0·4	+19·3	19·7	
			0·05	+29·0	+36·0	7·0	64
12	63	Water extract sterilised	0·05 heated	+ 2·4	+ 7·9	5·5	
			0·05	+12·4	+14·1	1·7	69
13	63	Water extract	0·05 heated	0·0	+14·9	14·9	
			0·05	+ 8·6	+14·1	5·5	63
14	63	Water extract	0·02 heated	+ 1·3	+15·8	14·5	No inhibition
			0·02	+ 2·9	+18·0	15·1	
15	Twort	Water extract	0·05 heated	+ 0·7	+ 9·3	8·6	
			0·05	+ 3·7	+10·8	7·1	17
16	63	Water extract	0·05 heated	− 0·9	+ 6·2	7·1	No inhibition
			0·05	+ 8·0	+24·2	16·2	
17	63	Water extract sterilised	0·05 heated	− 1·8	+ 3·7	5·5	No inhibition
			0·05	+ 6·3	+16·2	9·9	
18	63	Water extract	0·05 heated	+ 2·8	+ 8·0	5·2	
			0·05	+ 3·2	+ 7·6	4·4	15
19	63	Water extract	0·05 heated	+ 1·1	+ 4·1	3·0	No inhibition
			0·05	+ 4·8	+ 7·9	3·1	
20	63	F and W No amylase	0·05 heated	+ 8·2	+15·3	7·1	No inhibition
			0·05	+ 5·2	+20·6	15·0	
21	63	F and W No amylase	0·1 heated	− 1·6	+ 7·9	9·5	No inhibition
			0·1	+ 0·2	+13·8	13·6	
22	63	F and W No amylase	0·1 heated	− 0·8	+ 6·8	7·6	No inhibition
			0·1	+ 2·0	+11·6	9·6	

of starch was in all probability amylase. All extracts after that were tested for amylase. In three of the seven experiments (Nos. 20, 21 and 22) in which the pancreatic extract did not cause an inhibition of glycolysis, alcoholic extracts made according to Foster and Woodrow's directions were used. Each of these extracts was tested for amylase and gave a practically negative result. In another of the experiments (19) in which there was no inhibition, the aerobic glycolysis without pancreas was already very low, 3 mm.³ CO_2 being the production by 1 mg. of tissue in 1 hour as compared with an average of 12 mm.^{3} per hour. Discarding those experiments in which the alcoholic extracts contained no amylase, the results in Table I show that pancreatic extracts caused an inhibition in the aerobic glycolysis of cancer tissue in 15 out of 19 cases. The solution containing unheated pancreatic extract and no glucose generally gave off more CO_2 than did that containing heated extract. It is unfortunate that the smaller figure for glycolysis with pancreatic extract is obtained in some cases because the larger control is subtracted, but this does not apply to the malt diastase experiments nor to the anaerobic and chemical experiments to be given later which show that the lower glycolysis with unheated pancreatic extract is real.

Table II.

				mm.^{3} CO_2 per mg. per hour			
No. of exp.	Carcinoma	Inhibitor	cc.	Without glucose	With glucose	Aerobic glycolysis	Inhibition %
1	63	Malt diastase	0·05 heated	+ 2·4	+ 16·9	14·5	
			0·05	+ 2·8	+ 6·9	4·1	71
2	63	Malt diastase	0·1 heated	+ 3·3	+ 21·7	18·4	
			0·1	+ 5·6	+ 11·7	6·1	66
3	63	Malt diastase	0·1 heated	+ 4·8	+ 11·9	7·1	
			0·1	+ 3·8	+ 5·6	1·8	74
4	63	Malt diastase	0·1 heated	+ 4·0	+ 11·1	7·1	
			0·1	+ 3·8	+ 9·4	5·6	21
5	63	Malt diastase	0·05 heated	+ 0·3	+ 33·8	33·5	
			0·05	+ 4·8	+ 16·0	11·2	63
6	63	Malt diastase sterilised	0·05 heated	+ 2·5	+ 5·6	3·1	
			0·05	+ 2·8	+ 4·7	1·9	39
7	63	Malt diastase sterilised	0·1 heated	− 1·4	+ 11·3	12·7	
			0·1	+ 2·4	+ 5·6	3·2	74
8	63	Malt diastase sterilised	0·1 heated	+ 0·4	+ 9·4	9·0	
			0·1	+ 3·4	+ 8·9	5·5	39
9	63	Malt diastase half strength	0·05 heated	− 1·9	+ 3·0	4·9	No inhibition
			0·05	− 1·2	+ 4·6	5·8	
10	Twort	Takadiastase	0·1 heated	+ 3·2	+ 9·7	6·5	
			0·1	+ 8·6	+ 9·2	0·6	90
11	Twort	Takadiastase	0·1 heated	+ 1·9	+ 22·7	20·8	
			0·1	+ 14·0	+ 10·2	—	100
12	Twort	Takadiastase	0·1 heated	0·0	+ 10·4	10·4	
			0·1	+ 7·3	+ 12·4	5·1	51
13	63	Takadiastase	0·1 heated	+ 1·5	+ 7·6	6·1	
			0·1	+ 14·0	+ 13·5	—	100
14	63	Takadiastase	0·1 heated	+ 0·7	+ 6·8	6·1	
			0·1	+ 6·1	+ 8·0	1·9	69
15	63	Takadiastase	0·1 heated	+ 1·0	+ 12·3	11·3	
			0·1	+ 3·9	+ 11·9	8·0	29
16	63	Takadiastase	0·1 heated	+ 3·7	+ 8·1	4·4	
			0·1	+ 7·5	+ 10·9	3·4	22
17	63	Takadiastase	0·1 heated	+ 6·8	+ 13·1	6·3	No inhibition
			0·1	+ 5·5	+ 14·4	8·9	
18	63	Salivary gland	0·1 heated	+ 6·0	+ 23·4	17·4	
			0·1	+ 5·4	+ 12·2	6·8	61
19	63	Salivary gland	0·1 heated	+ 2·6	+ 9·2	6·6	
			0·1	+ 2·8	+ 7·2	4·4	33
20	63	Salivary gland	0·1 heated	+ 2·2	+ 10·4	8·2	
			0·1	+ 4·4	+ 9·4	5·0	39
21	63	Salivary gland	0·1 heated	+ 0·8	+ 7·9	7·1	
			0·1	+ 3·2	+ 9·6	6·4	9
22	63	Salivary gland	0·1 heated	+ 0·2	+ 4·7	4·5	No inhibition
			0·1	0·0	+ 5·0	5·0	

Since Case and McCullagh had shown the inhibiting factor for muscle glycolysis to be present in other extracts containing amylase, we tried the effect of such extracts on the aerobic glycolysis of cancer tissue. Malt diastase and takadiastase extracts were made by extracting 0·5 g. dried commercial preparation with 5 cc. water for 1 hour, filtering and adjusting to about p_H 7·6. The salivary gland preparation was made as follows. The submaxillary gland of an ox was minced, ground with acetone twice, filtered, ground with ether twice, filtered and dried in air. 0·2 g. was extracted in 8 cc. water.

The effects of malt diastase, takadiastase and salivary gland extracts on the aerobic glycolysis of cancer tissue are shown in Table II.

Table II shows that in 22 experiments in which malt diastase, takadiastase or dried salivary gland was used, there were 19 cases in which the presence of one or other of these preparations caused an inhibition in the aerobic glycolysis of cancer tissue.

Similar experiments using pancreatic extracts were then made anaerobically. The experiments were carried out in the manner already described except that the gas mixture used for filling the flasks consisted of 95 % nitrogen and 5 % CO_2. The results are given in Table III. The pancreatic extract used in these experiments was prepared according to the method of Ronzoni, Glaser and Barr.

The tissue used in all the experiments in this table was mouse carcinoma 63.

Table III.

No. of exp.	Pancreatic extract cc.	mm.^{3} CO_2 with glucose	Inhibition %
1	0·1 heated	29·5	
	0·1	21·1	28
2	0·1 heated	11·1	
	0·1	1·2	89
3	0·1 heated	15·8	
	0·1	3·3	79
4	0·1 heated	6·5	
	0·1	1·0	84
5	0·1 heated	24·0	
	0·1	13·0	46
6	0·1 heated	10·2	
	0·1	7·5	26
7	0·1 heated	11·7	
	0·1	0·4	96

It can be seen from Table III that pancreatic extract made according to Ronzoni, Glaser and Barr's directions caused an inhibition (ranging from 26 to 96 %) in the anaerobic glycolysis of cancer tissue in all of the seven experiments.

Several experiments were then carried out on a larger scale, in which the lactic acid formed was estimated directly by the chemical method of Friedemann, Cotonio and Shaffer [1927]. The pancreatic extracts for these experiments were made according to Ronzoni, Glaser and Barr, a number of different preparations being used. A control was always done using Ringer solution and pancreatic extract without tissue. The iodine value for this was subtracted from that obtained when a similar solution contained tissue. Also a control experiment was always made with tissue in Ringer with no added sugar to allow for the lactic acid from the tissue alone. The reading obtained from this control was subtracted from that obtained for the sugar-containing solutions both with and without pancreatic extract. 14 cc. bicarbonate Ringer, 5 cc. pancreatic extract, or 5 cc. water (or in some cases 5 cc. heated extract) and 1 cc. glucose solution (of such strength that the final glucose concentration was 0·4 %) were put into a flask and a weighed amount of cancer tissue slices added. (A number of tumours were sliced thinly, the slices drained quickly on filter paper, and, having been picked at random from the mixed tumours, were weighed, about 0·4 to 0·5 g. being added to each flask.) The flasks were filled with nitrogen containing 5 % CO_2, stoppered and shaken in a water-bath at 37° for 2 hours. Before the estimation of lactic acid, proteins were removed by trichloroacetic acid and sugars by copper sulphate and calcium hydroxide. Table IV gives the results of these experiments. P.E. designates pancreatic extract.

Table IV.

No. of exp.	Cancer tissue	Mg. lactic acid formed per g. dry wt. tissue		Inhibition %
1	63	No P.E.	31·5	
		With P.E.	12·0	61
2	Human fibro-	No P.E.	32·8	
	sarcoma of leg	With P.E.	0·0	100
3	63	No P.E.	16·5	
		With P.E.	9·5	42
4	63	No P.E.	48·7	
		With P.E.	22·7	53
5	63	No P.E.	18·0	
		With P.E.	0·2	99
6	63	No P.E.	27·5	
		With P.E.	22·0	20
7	63	No P.E.	46·5	
		With P.E.	19·0	59
8	63	No P.E.	37·0	
		With P.E.	30·0	19
9	63	No P.E.	48·0	
		With P.E.	6·0	87
10	63	No P.E.	61·0	
		With P.E.	20·0	67
11	63	No P.E.	80·5	
		With P.E.	26·0	67
12	63	No P.E.	37·5	
		With P.E.	7·2	80
13	63	No P.E.	30·0	
		With P.E.	3·0	90
14	63	No P.E.	30·4	
	+ fructose	With P.E.	6·9	77
15	63	No P.E.	22·5	
	+ fructose	With P.E.	6·5	71

Table IV shows that in the 13 experiments in which glucose was used the pancreatic extract in all cases caused an inhibition in the amount of lactic acid formed, ranging from 20 to 100 %. Warburg has shown that cancer tissue can use fructose also as a precursor of lactic acid. In the last two experiments in Table IV, 0·4 % fructose was used instead of glucose and it can be seen that pancreatic extract inhibits this glycolysis also.

Barr, Ronzoni and Glaser [1928] quote a total of 21 manometric experiments on the glycolysis of cancer tissue in the presence of pancreatic extract, 8 cases of inhibition occurring, ranging from 6 to 37 %. They also give 6 chemical experiments in 2 of which inhibition occurred, to the extent of 5 and 29 % respectively. They conclude that pancreatic extract causes no appreciable inhibition in the glycolysis of cancer tissue. If all the experiments in the previous four tables are considered together (not counting the 3 in which the pancreatic extract had no amylase), there is a total of 63, with only 4 where no inhibition occurred. We feel justified, then, in stating that some factor, presumably amylase, common to pancreatic extract, malt diastase, takadiastase and dry salivary gland, does cause an inhibition in the glycolysis of the cancer tissue used in this laboratory.

The question now arises as to the mechanism by which these various extracts bring about inhibition. McCullagh has shown that the substance which brings about the inhibition in muscle glycolysis when starch is used is amylase. After the publication of this work, tests for amylase were always made on the various extracts used in our work, and we found that extracts containing no amylase gave no inhibition in the glycolysis of cancer tissue. It is difficult to make any accurate comparison using cancer tissue between the degree of inhibition caused by various preparations and their amylase content, because the use of slices of tissue, rather than the homogeneous tissue extract which McCullagh used from muscle, involves an experimental error which invalidates any but fairly large differences. Barr, Ronzoni and Glaser made an extract of cancer tissue but found it to be glycolytically inactive. It is therefore, at the moment, impossible to work with a more homogeneous source of the glycolytic enzyme from cancer tissue than is provided by slices of tissue. We assume, then, for the time being, that the factor inhibiting glycolysis in cancer tissue is amylase. McCullagh claims that amylase inhibits the glycolysis of starch because it prevents the formation of hexosephosphate which is known to be an intermediate product in the breakdown by muscle of starch to lactic acid. Barr, Ronzoni and Glaser, who added phosphate to bicarbonate-Ringer, observed no increase in the lactic acid formation by cancer tissue, and hence they assumed that glucose, when acted upon by cancer tissue, did not go through a phosphate stage. This assumption was supported by their inability to observe any changes in inorganic phosphorus during the course of glycolysis in cancer tissue. They therefore explain their observations that in the majority of cases pancreatic extract did not inhibit the glycolysis of cancer tissue, by agreeing with McCullagh as to the function of the inhibiting factor, namely that in muscle it prevents the formation of hexosephosphate. If, in cancer metabolism, hexosephosphate plays no part, on the above assumption no inhibition of glucose glycolysis would be expected. But, as the previous tables have shown, in 95 % of our experiments pancreatic extract (or some other amylase-containing extract) did cause an inhibition of glycolysis in cancer tissue. We then made a number of experiments, using hexosediphosphate and hexosemonophosphate as substrates for glycolysis instead of glucose. Barium hexosediphosphate and barium hexosemonophosphate were very kindly given to us by Dr R. Robison, who informed us that the hexosemonophosphate was of the usual mixed aldose-ketose type, obtained by fermentation with yeast juice and purified by precipitation as basic lead salt.

We also wish to express our thanks to Bayer Products, Ltd., for very kindly presenting us with a quantity of calcium hexosediphosphate. The hexosephosphate solutions were made up so that they were equivalent to a final concentration of 0·4 % hexose, thus making them comparable with solutions containing 0·4 % glucose. Calcium hexosediphosphate was dissolved in the molecular equivalent amount of sodium oxalate, and the calcium oxalate centrifuged off, the supernatant liquid always being tested to ensure freedom from oxalate. The analysis provided by Bayer Products, Ltd. of the calcium hexosediphosphate showed that it contained 29 % water, an allowance for which was made in the quantities used. Barium hexosemonophosphate was treated with the molecular equivalent amount of Na_2SO_4, centrifuged and tested for freedom from Ba. The hexosediphosphate solution was prepared in the same way. Several experiments were done anaerobically using the manometric method already described. The results of these experiments are given in Table V. Carcinoma 63 was used. P.E. represents pancreatic extract.

Table V.

No. of exp.	Substrate	mm.³ CO_2 per mg. dry wt. tissue per hour
1	Glucose	29·5
	„ + P.E.	21·1
	Hexosediphosphate	3·9
	„ + P.E.	0·8
2	Glucose	11·1
	„ + P.E.	1·2
	Hexosediphosphate	2·6
	„ + P.E.	4·4
3	Glucose	15·8
	„ + P.E.	3·3
	Hexosediphosphate	0·0
	„ + P.E.	0·0
4	Glucose	6·5
	Hexosediphosphate	0·0
5	Glucose	10·2
	Hexosediphosphate	0·0
	Hexosemonophosphate	0·0
6	Glucose	11·7
	„ + P.E.	0·4
	Hexosemonophosphate	0·8
	„ + P.E.	1·5

Several experiments, in which the lactic acid was determined chemically, were carried out using these substrates. Their results are given in Table VI. Carcinoma 63 was used.

Table VI.

No. of exp.	Substrate	Mg. lactic acid per g. dry wt. tissue
1	Glucose	55·0
	Hexosediphosphate	0·0
2	Glucose	30·0
	,, +P.E.	3·0
	Hexosediphosphate	1·7
	,, +P.E.	0·0
	Hexosemonophosphate	0·0
	,, +P.E.	0·0
3	Fructose	22·5
	,, +P.E.	6·5
	Hexosediphosphate	7·0
	,, +P.E.	0·0
	Hexosemonophosphate	2·5

The experiments in Tables V and VI show that cancer tissue cannot use either hexosediphosphate or hexosemonophosphate to any appreciable extent as a source of lactic acid. Starch was tried also as a substrate for cancer glycolysis but the tumour tissue was unable to act upon it, as Barr, Ronzoni and Glaser have also found. It is clear that the hypothesis that pancreatic extract inhibits glycolysis by preventing the formation of hexosephosphate is not applicable at any rate to cancer tissue, since the latter cannot form lactic acid from either the diphosphate or the monophosphate when they are supplied.

The possibility that the inhibition might be a simple adsorption of glucose by the unheated extract is rendered unlikely by the result of the following experiment in which doubling the concentration of glucose, leading to an increase of free sugar in the solution, produced no appreciable change in the inhibition brought about by a given amount of pancreatic extract. Carcinoma 63 was used.

Substrate	mm.^{3} CO_2 per mg. dry wt. tissue per hour	Inhibition %
Glucose 0·4 %	34·0	
,, ,, +P.E.	13·0	46
Glucose 0·8 %	25·6	
,, ,, +P.E.	14·6	43

As yet, then, we have no explanation to offer for the mechanism by which pancreatic extract inhibits the lactic acid formation in cancer tissue.

As a result of this work we decided to investigate McCullagh's theory of the cause of inhibition by amylase of glycolysis in muscle, according to which pancreatic extract inhibits the formation of lactic acid by preventing the intermediate formation of hexosephosphate. We have already shown that this theory does not explain the inhibition produced by pancreatic extract on the glycolysis in cancer tissue. We now tried the effect of pancreatic extract on the soluble muscle enzyme system of Meyerhof [1926], to which hexosephosphate had already been added. The results are given in Table VII. For these experiments, the soluble muscle enzyme was prepared according to the directions of McCullagh. The quantities used in each flask were 8 cc. of muscle extract, 3 cc. phosphate buffer (made according to McCullagh), 4 cc. pancreatic extract (Ronzoni, Glaser and Barr's), 3 cc. 2·4 % substrate (starch, glucose, or hexosephosphate which was 2·4 % with respect to hexose), and 2 cc. water or yeast activator (made according to Meyerhof [1927]). 5 cc. of the contents of each flask were removed immediately into 1 cc. of 20 % trichloroacetic acid, these solutions serving as controls. The remainder was incubated for 4 hours at 37°, and then 3 cc. of 20 % trichloroacetic acid were added. After filtering off the proteins, the sugars, starch and hexosephosphate were precipitated in an aliquot neutralised portion by $CuSO_4$ and $Ca(OH)_2$. This method precipitates not only glucose, but, as was shown by Embden and Zimmermann [1924], other carbohydrates including hexosephosphates. The lactic acid in an aliquot portion of the final filtrate was estimated by the method of Friedemann, Cotonio and Shaffer. Results are expressed in mg. of lactic acid produced by 10 cc. of soluble muscle enzyme. Controls were always done in which no substrate was added as there proved to be always a small formation of lactic acid with no added substrate. These control figures were subtracted from those obtained with solutions to which substrate was added. In Exp. 1, Table VII, barium hexosediphosphate was used. In the other experiments in this table, the calcium salt was used. P.E. designates pancreatic extract.

For Exp. 12 in Table VII, the water extracts of pancreas, takadiastase and malt diastase were made by extracting 2 g. of dry preparation with 30 cc. water for 2 hours, filtering and neutralising. The saliva was diluted 1 : 5.

In some of the experiments in which glucose was used as a substrate, an activator prepared from yeast by the method of Meyerhof was added. Meyerhof has shown that the soluble muscle enzyme system has in large part lost the power of changing glucose to lactic acid. This power he shows to be restored when yeast activator is added.

The effect of pancreatic extract on glycolysis in the soluble muscle enzyme system is seen from the results in Table VII to be as follows. (1) When starch is the substrate, inhibition is brought about; (2) when hexosediphosphate is the substrate, inhibition is again brought about; but (3) when hexosemonophosphate or (4) glucose plus yeast activator are the substrates, no appreciable inhibition occurs.

The fact that pancreatic extract inhibits glycolysis in the soluble muscle enzyme system not only when starch is the substrate but also when hexosediphosphate is used, shows that the inhibiting factor does not act by preventing the formation of hexosediphosphate, because when hexosediphosphate is added to the system, inhibition still occurs. The weight of evidence seems

Table VII.

No. of exp.	Substrate	Mg. lactic acid per 10 cc. muscle extract	Inhibition %
1	Starch	27·3	
	,, + P.E.	22·6	17
	Glucose	4·9	
	,, + P.E.	0·0	100
	Hexosediphosphate	16·6	
	,, + P.E.	3·7	77
2	Starch	11·5	
	,, + P.E.	8·2	28
	Glucose	2·5	
	,, + P.E.	0·0	100
	Hexosediphosphate	9·0	
	,, + P.E.	0·0	100
3	Starch	16·0	
	,, + P.E.	0·6	91
	Glucose	3·1	
	,, + yeast activator	7·2	
	,, + yeast activator + P.E.	7·2	0
	Hexosediphosphate	8·9	
	,, + P.E.	3·5	60
4	Starch	7·2	
	,, + P.E.	0·9	87
	Hexosediphosphate	24·8	
	,, + P.E.	13·9	44
5	Starch	4·3	
	,, + P.E.	0·0	100
	Glucose	0·7	
	,, + P.E.	0·0	
	,, + yeast activator	3·3	
	,, + yeast activator + P.E.	5·0	0
	Hexosediphosphate	5·2	
	,, + P.E.	0·0	100
6	Starch	11·3	
	,, + P.E.	2·1	81
	Hexosediphosphate	17·8	
	,, + P.E.	8·8	50
	Hexosemonophosphate	20·5	
	,, + P.E.	19·2	6
7	Hexosemonophosphate	17·6	
	,, + P.E.	19·3	0
8	Starch	8·1	
	,, + P.E.	1·8	77
	Hexosediphosphate	18·9	
	,, + P.E.	9·0	52
	Hexosemonophosphate	13·0	
	,, + P.E.	13·5	0
9	Starch	15·6	
	,, + P.E.	8·6	45
	Hexosediphosphate	17·6	
	,, + P.E.	10·7	39
	Hexosemonophosphate	23·0	
	,, + P.E.	22·7	1
10	Starch	2·7	
	,, + P.E.	0·7	74
	Glucose + yeast activator	8·8	
	,, + yeast activator + P.E.	9·7	0
	Hexosediphosphate	6·0	
	,, + P.E.	1·8	70
	Hexosemonophosphate	17·3	
	,, + P.E.	14·9	13

Table VII (*cont.*)

No. of exp.	Substrate	Mg. lactic acid per 10 cc. muscle extract	Inhibition %
11	Starch	16·6	
	„ +P.E.	7·0	58
	„ +yeast activator	12·4	
	„ +yeast activator + P.E.	7·6	38
	Hexosediphosphate	6·4	
	„ +P.E.	1·0	84
	„ +yeast activator	11·0	
	„ +yeast activator + P.E.	0·0	100
	Hexosemonophosphate	13·3	
	„ +P.E.	15·8	0
12	Starch	14·9	
	+P.E. (R.G. and B.)	0·0	100
	+P.E. (water)	0·0	100
	+takadiastase	2·3	84
	+malt diastase	0·0	100
	„ +saliva	11·9	20
	Hexosediphosphate	18·9	
	+P.E. (R.G. and B.)	5·3	72
	+P.E. (water)	0·0	100
	+takadiastase	1·3	93
	+malt diastase	2·5	86
	+saliva	5·4	71

now, however, to point to hexosemonophosphate as being the normal precursor of lactic acid in muscle [Embden and Zimmermann, 1927; Meyerhof and Lohmann, 1927; Pryde and Waters, 1927]. Table VII shows that when hexosemonophosphate is added to the soluble muscle enzyme system, pancreatic extract no longer causes an inhibition in the formation of lactic acid. This observation is compatible with McCullagh's theory that the inhibiting power of the pancreatic extract is due to the prevention of esterification. When a substrate like glycogen is used from which hexosephosphate must be synthesised, the pancreatic extract causes inhibition in the amount of lactic acid formed, but when monophosphate itself is used, in which case obviously no synthesis is required, pancreatic extract causes no inhibition in lactic acid formation. Case and McCullagh showed that when glucose with yeast activator was added to the muscle enzyme, pancreatic extract caused no inhibition in lactic acid formation. Our own results (Table VII) confirm this observation. Meyerhof has shown that glucose with yeast activator and the muscle enzyme forms in part a labile hexosephosphate which is broken down to lactic acid. In other words, an esterification of glucose and phosphate must take place before lactic acid is formed. Case and McCullagh believe esterification to be impossible in the presence of pancreatic extract, yet their results and our own show that pancreatic extract in no way inhibits the lactic acid formation (and hence, according to Meyerhof, the hexosephosphate formation) from glucose acted upon by the muscle enzyme in the presence of yeast activator. Case and McCullagh state that since the inhibiting factor is amylase, and amylase does not act on glucose, no inhibition in lactic acid formation from glucose was to be expected. This deduction is difficult to understand, because the function they have attributed to amylase, namely, the inhibition of esterification, is not one which it could exert on starch (its known substrate) since starch does not combine directly with phosphorus to make hexosephosphate. Meyerhof pictures that in the breakdown of starch there is first formed a labile hexose which then becomes esterified. It seems unlikely that the inhibiting factor would prevent esterification of this labile hexose from starch when it does not prevent esterification of glucose in the presence of its activator. It might appear at first sight possible that the yeast activator itself prevented the inhibiting action of pancreatic extract. but that such is not the case is clear from the results of Exp. 11, Table VII, where the addition of yeast activator to a muscle enzyme system acting on starch in the presence of pancreatic extract did not prevent the usual inhibition by the pancreatic extract from taking place. In this experiment, yeast activator was also added to the muscle enzyme system in which hexosediphosphate was the substrate. The inhibition produced by the pancreatic extract was not diminished by the presence of the activator. These observations led us to think that the inhibiting factor in the various extracts studied, while presumably amylase as Case and McCullagh believe, was not causing inhibition in lactic acid formation in muscle in the way they suggest, namely, by preventing esterification. McCullagh's evidence for his theory that hexosephosphate cannot be formed in the presence of amylase is, "When the formation of lactic acid was prevented by the use of the pancreatic factor, the total reducing power after acid hydrolysis sufficient to break down starch but not hexosephosphate was not changed. The hexosephosphates differ from glucose in their reducing power....These results, therefore, suggested that hexosephosphates were not being formed in the presence of pancreatic extract." Ronzoni, Glaser and Barr show a large increase in the amount of free reducing substances in the presence of the pancreatic factor. This increase in free reducing sugars is much greater in the presence of pancreatic extract than in its absence. These two facts considered together, first, that there is no loss of total carbohydrate and second, that there is a very large increase in free sugar in the presence of the pancreatic extract, might, from one point of view, be considered as evidence that. after the breakdown of glycogen to free sugar, the pancreatic factor exerts its activity, preventing this free sugar from becoming esterified. Hence the free sugar accumulates and the total carbohydrate remains the same. These observations, however, can be equally well explained on the hypothesis suggested below. The second piece of evidence advanced by Case and McCullagh that the pancreatic factor prevents esterification is the fact that in the presence of the pancreatic factor, inorganic phosphorus does not disappear, but in fact increases, whereas in a muscle enzyme system free from pancreatic extract, inorganic phosphorus decreases, pointing to the formation of a hexosephosphate. We consider that this evidence does not prove that the inhibiting power of pancreatic extract is due to the prevention of esterification of hexose and phosphate. We suggest that a simpler explanation of the experimental observations can be based on the well-known fact that amylase breaks down starch to maltose. Meyerhof has shown that maltose yields only a very small amount of lactic acid when acted upon by the muscle enzyme system. Hence, the effect to be expected on adding pancreatic extract to a muscle enzyme system acting on starch would be a decreased lactic acid formation. Embden and Haymann [1924] have shown that maltose when added to muscle does not increase the formation of hexosephosphate; in other words, maltose will not link up with inorganic phosphorus in the presence of the muscle enzyme to form a hexosephosphate. This means, then, that in the presence of the pancreatic extract there would be no appreciable change in total free carbohydrates because no appreciable amounts of lactic acid or of hexosephosphate were being formed. But there would be a large increase in free sugar because starch was being broken down to maltose. In the uninhibited muscle system, starch is broken down to a labile hexose which is quickly esterified so that there is little increase in free sugar. Again, let us examine the inorganic phosphorus results of Case and McCullagh and Ronzoni, Glaser and Barr. In the uninhibited muscle system the amount of inorganic phosphate is reduced because hexosephosphate is built up more quickly than it is broken down. In the presence of pancreatic extract, inorganic phosphate does not disappear because maltose, which the amylase has formed from starch, does not link up with phosphorus to form hexosephosphate. In this manner, the results obtained by Case and McCullagh, Ronzoni, Glaser and Barr, and ourselves can be explained without assuming any unknown action of amylase on starch. Also this explanation, unlike that of McCullagh, is compatible with the results obtained with glucose and yeast activator.

Our results have shown that the production of lactic acid from hexosediphosphate is also inhibited by the pancreatic factor. Is this inhibition, like that of the starch, also due to amylase? Exp. 12, Table VII, shows the results of an attempt to find out whether the inhibition of lactic acid formation from these two substances was caused by the same factor. Several different amylase preparations were used and their effects on the glycolysis of starch and hexosediphosphate are in the main roughly parallel, so that, unless another factor were present in the various extracts in about the same proportion as amylase, the inhibition of the glycolysis of hexosediphosphate appears to be due to the same factor that causes the inhibition of the glycolysis of starch.

At present we cannot explain the mechanism by which pancreatic extract causes an inhibition in lactic acid formation from hexosediphosphate.

Several of Ronzoni, Glaser and Barr's experimental facts can be interpreted from our own results. They found, as did Foster and Woodrow, that with muscle itself, the pancreatic extract caused only 40–60 % inhibition. Ronzoni, Glaser and Barr show that this cannot wholly be explained by insufficient permeation of the muscle by the pancreatic extract and assume that the incomplete inhibition is due to the presence of some preformed precursor of lactic acid. The precursor of lactic acid in muscle has been shown by various workers already quoted to be hexosemonophosphate. Our results show that hexosemonophosphate is not inhibited by the pancreatic extract and hence the incomplete inhibition obtained when pancreatic extract acted on muscle can be explained. Again, Ronzoni, Glaser and Barr give experiments in which the muscle enzyme system was for a time allowed to form hexosephosphate and then the inhibiting factor was added. After incubation, there was found to be some lactic acid formed in spite of the presence of the inhibitor. They explain that this is from the hexosephosphate formed before the inhibitor was added. This again fits in with our results in which the glycolysis of hexosemonophosphate is not inhibited by pancreatic extract. We suggest that what happens in the muscle enzyme system under various conditions is as follows:

Starch → Hexosemonophosphate → Lactic acid
↓ Pancreatic factor inhibits by forming
Maltose

Hexosediphosphate → Lactic acid
↓ Pancreatic extract inhibits possibly by forming
Free phosphate and a hexose not acted upon by muscle

Hexosemonophosphate → Lactic acid
↓ Pancreatic extract has no effect

Glucose + yeast activator → Hexosemonophosphate → Lactic acid
↓ Pancreatic extract has no effect

It is impossible at the moment to fit the cancer system into this scheme. as, in cancer, glucose glycolysis is inhibited by the pancreatic factor; also in muscle, glucose goes through a phosphate stage and in cancer it probably does not. It must be borne in mind, however, that in the muscle experiments an extract is used, while in the cancer experiments a slice of tissue is used. We are, however, able to say that in glycolysis by cancer tissue the pancreatic factor does not inhibit the formation of lactic acid by preventing the formation of hexosephosphates since the cancer tissue is unable to utilise such compounds when added.

The results obtained with cancer tissue indicate that the processes leading to the formation of lactic acid by cancer tissue are less complex than those in muscle. Muscle can form lactic acid not only from glucose but also from starch, hexosediphosphate and hexosemonophosphate, whereas cancer can use none of these substances except glucose as a source of lactic acid. Meyerhof has shown that the different stages of glycolysis in muscle are brought about by different enzymes. Our results indicate that cancer tissue contains an enzyme for glycolysing glucose but no enzymes for glycolysing starch and the hexosephosphates. The mechanism in cancer tissue by which glucose is changed to lactic acid is, according to our results, different from that in muscle. In the latter, glucose goes through a hexosephosphate stage before lactic acid is formed, whereas in cancer the evidence is against the intermediate formation of a hexosephosphate in the production of lactic acid from glucose.

SUMMARY.

1. The aerobic and anaerobic glycolysis of cancer tissue is inhibited by pancreatic extract.

2. Other amylase-containing extracts made from malt diastase, taka-diastase and salivary gland also inhibit glycolysis in cancer tissue.

3. Cancer tissue is unable to form lactic acid from hexosediphosphate or hexosemonophosphate.

4. In the soluble muscle enzyme system, the production of lactic acid from hexosediphosphate is inhibited by pancreatic extract, while the production of lactic acid from hexosemonophosphate is not inhibited by pancreatic extract.

We are indebted to the Yorkshire Cancer Research Fund for the expenses incurred in carrying out this work.

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XXI. THE DENATURATION OF PROTEINS.

VI. THE TITRATION OF THE BASIC AND ACIDIC GROUPS IN EGG-ALBUMIN.

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INTRODUCTION.

THE present paper contains an account of work carried out to determine, with a greater degree of precision than has been hitherto possible, whether or no there is any change in the number of free amino- and carboxyl groups in the protein on denaturation.

Let us first consider the work carried out by P. S. Lewis [1927] in this laboratory in which he determined the number of free carboxyl and amino-groups in haemoglobin before and after denaturation. His method was to add a known amount of standard acid or alkali to a definite volume of protein solution of known strength, and then to measure the p_H. The p_H was then plotted against the amount of acid or alkali added, for denatured and undenatured protein respectively. He found that the curves were identical, and so concluded that there was no change in the number of free amino- and carboxyl groups as a consequence of denaturation. However, in the light of later work by Cubin [1929], also in this laboratory, on denaturation at low temperatures and low p_H values, it appears that the concentration of acid in most of P. S. Lewis's experiments was sufficient to denature the haemoglobin during the time taken in carrying out the titration process itself. Thus from Cubin's results of the rate of denaturation at 25°, the temperature at which P. S. Lewis performed his titrations, it is found that denaturation takes place below p_H 4·0 almost instantaneously. The titration curve obtained by P. S. Lewis depends on eight points, three of which are above p_H 4 and five below. The titration is complete at a p_H of about 2·2. Hence we must conclude that in his measurements the amount of acid added for complete titration (obtained from the more acid end of the curve) was the same because he was in each case dealing with denatured protein. This fact has been confirmed by the writer in the following way. A haemoglobin solution of the strength used by P. S. Lewis was kept at 25° in the same quantity of 0·096 N acid and for the same length of time as employed by him. The solution was rapidly cooled to 0°. By addition of ammonia the p_H was adjusted to the isoelectric point when the protein flocculated. Hence the titration curves obtained by P. S. Lewis below p_H 4 (the most important region) were identical because he was in each case inadvertently titrating denatured material.

In the case of his alkali titrations it is not possible to speak with such certainty. Assuming, however, that the action of alkali at a certain distance on one side of the neutrality point is just as great as that of acid at the same distance on the other side (certainly true for p_H values close to the neutrality point), we infer that over part of the titration range the material was denatured in both cases. It is only fair to this investigator to point out that this criticism of his titration conditions only becomes possible in the light of the later work by Cubin.

As regards the chemical nature of denaturation several possibilities, plausible on general grounds, have been criticised on the score that they would involve a change in the number of free carboxyl and amino-groups in the protein molecule on denaturation. Hence it is obviously of considerable importance to attempt to determine whether this change does or does not occur. The only means of doing this for haemoglobin would be by working at sufficiently low temperatures. The lowest temperature at which it would be possible to work is 0°, and the writer has calculated the values of the velocity constants of denaturation of haemoglobin at this temperature over the significant range, viz. $p_H < 4$, assuming the critical increment 11,000 calories obtained by Cubin for 18° to 25° to be valid for the range 18° to 0°. It is found that the amounts of acid used are sufficient rapidly to denature the protein at this temperature during the time of the experiment. Thus at p_H 3·5, 50 % of the haemoglobin would be denatured in ten minutes, whilst at p_H 3·0 denaturation is practically instantaneous.

It appears possible that another protein, egg-albumin, might be a more suitable material to use in these experiments, in view of the fact that denaturation in the acid region is much less easily brought about than it is in the case of haemoglobin. Accordingly the writer has carried out these titrations using crystallised egg-albumin. The egg-albumin was prepared by the method of Hopkins and Pinkus as modified by Sørensen [1917]. The estimation of the albumin content was made by the method of Devoto as given by Hopkins [1900].

TITRATION OF EGG-ALBUMIN WITH ACID.

The method employed in these experiments was that used by Harris [1923, 2, 3; 1924] for the titration of various amino-acids and complex electrolytes, and adopted by P. S. Lewis [1927] for haemoglobin.

The method is to determine the p_H reached when a known volume of standard acid is added to a definite volume of protein solution of known strength. A series of readings is taken, corresponding to the addition of varying volumes of the acid. Thus a curve can be plotted of p_H attained against volume of acid added. In a similar way a curve can be obtained of the p_H attained by the solvent against volume of acid added. This serves as a blank correction curve. Subtracting the volume of acid required to bring the solvent to a certain p_H from that required to bring both solvent and protein to this p_H, one obtains the volume of acid combined with the protein. The curve obtained by plotting p_H against acid combined with protein at a series of p_H values is asymptotic to the p_H axis when sufficient acid has been added to complete the titration. Experiments of this nature were performed using undenatured and denatured egg-albumin respectively. The titration in each case starts from the neutrality point of water.

All measurements were carried out in a thermostat regulated to 25°. In order to guard against denaturation as a result of prolonged exposure to acid, each point on the curve was obtained as the result of a separate experiment. Each result shown is the mean value of several (generally four) readings. The denaturation was performed by warming the solution (at p_H 6·99 in order to prevent change of p_H due to hydrolytic scissions [cf. P. S. Lewis, 1926]) in a corked flask in the steam of a briskly boiling water-bath. After denaturation was complete the cooled flask was tilted in order to collect the moisture which had condensed on the sides of the vessel.

The p_H was determined by the quinhydrone electrode, following the technique of Corran and Lewis [1924], the cell employed being of the type:

Gold foil | Solution under test saturated with quinhydrone | Saturated KCl | Normal KCl saturated with calomel | Mercury

The solution used contained 1 g. of egg-albumin and 0·203 g. of ammonium sulphate per 100 cc. The blank solution contained 0·203 g. of ammonium sulphate per 100 cc. The acid used for titration was 0·0905 *N* sulphuric acid.

The p_H readings are accurate to p_H 0·01, and the titres to 0·005 cc.

The following are the results obtained (Tables I, II, III and Fig. 1).

Table I. *Titration of 10 cc. 1 °/₀ egg-albumin with 0·0905* N *sulphuric acid at 25°.*

Acid added to 10 cc. solution cc.	Mean p_H of blank	Mean p_H undenatured	Mean p_H denatured
0·00	6·96	6·94	6·95
0·05	4·04	—	—
0·10	3·41	5·68	5·69
0·20	3·04	4·78	4·76
0·30	2·79	4·12	4·13
0·50	2·60	3·51	3·53
0·75	2·42	3·08	3·10
1·00	2·27	2·72	2·71
1·50	2·13	2·33	2·33

TITRATION OF EGG-ALBUMIN WITH ALKALI.

The method employed in this section was the formaldehyde method of Sørensen [1907] for the titration of amino-acids. Normally the titration with alkali has to be carried to a very alkaline end-point in order to obtain complete titration. The addition of a certain amount of formaldehyde before titration enhances the acidic nature of the protein, and titration is complete at a much lower p_H. Harris [1924] attributes this to the combination of the amino-acids with the formaldehyde to give methylene-imino-derivatives. As an alternative to formaldehyde a fairly concentrated solution of alcohol can be used in order to enhance the acidic nature of the protein. Owing to the fact that alcohol acts as a powerful denaturing agent this method cannot be adopted here.

Table II. *Corrected titres for undenatured egg-albumin.*

p_H	Titre (cc.)	Blank (cc.)	Corrected titre
6·94	0·00	0·00	0·00
5·68	0·10	0·008	0·092
4·78	0·20	0·026	0·174
4·12	0·30	0·048	0·252
3·51	0·50	0·092	0·408
3·08	0·75	0·183	0·567
2·72	1·00	0·363	0·637
2·33	1·50	0·860	0·640

Table III. *Corrected titres for denatured egg-albumin.*

p_H	Titre (cc.)	Blank (cc.)	Corrected titre
6·95	0·00	0·00	0·00
5·69	0·10	0·008	0·092
4·76	0·20	0·026	0·174
4·13	0·30	0·047	0·253
3·53	0·50	0·090	0·410
3·10	0·75	0·177	0·573
2·71	1·00	0·365	0·635
2·33	1·50	0·860	0·640

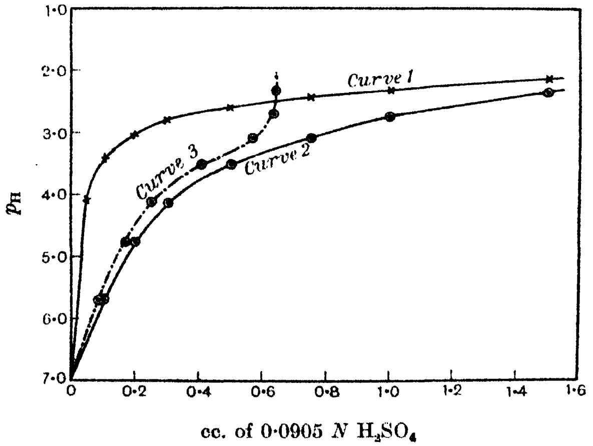

Fig. 1. Titration of egg-albumin with acid.

Curve 1, titration of solvent alone.
Curve 2, titration of undenatured and denatured albumin.
Curve 3, corrected titre for undenatured and denatured albumin.

It is known that in the presence of ammonium salts formaldehyde forms a mixture of methylamines, producing at the same time acids [Werner, 1917]. Thus with ammonium sulphate we get:

$$4CH_2O + (NH_4)_2SO_4 \rightarrow 2CH_3.NH_2 + 2H.COOH + H_2SO_4$$
$$2CH_2O + CH_3.NH_2 \rightarrow (CH_3)_2.NH + H.COOH$$

etc.

Hence it is not possible accurately to perform formaldehyde titrations in the presence of ammonium salts. In the present case it is not feasible to dialyse sufficiently long to remove all the ammonium salts. Although realising that true titration curves cannot be obtained by this method, the writer has performed the titration experiments to see if any noticeable difference in results occurred when using denatured in place of undenatured albumin.

In order to perform the titrations it is necessary to determine the p_H of a solution containing protein, salt, and formaldehyde when varying amounts of alkali are added. In order to guard against denaturation by alkali a fresh solution was made up before each reading. The glass electrode [Kerridge, 1925] was employed for measuring the p_H. If e_1 and e_2 are the E.M.F. measured using standard solution and solution of unknown p_H respectively, then

$$p_H = 3\cdot97 + \frac{e_2 -}{0\cdot58}\,.$$

In general the results are accurate to 0·02–0·03 p_H. The titres are accurate to 0·005 cc.

By titrating against the solvent alone (*i.e.* water, ammonium sulphate and formaldehyde) a blank correction curve was obtained. Hence, by subtraction, the volume of alkali required to bring the protein to a definite p_H can be obtained. The results are given in Tables IV, V and VI, and the accompanying curves (Fig. 2) drawn from them.

Table IV. *Titration by 0·1357* N *sodium hydroxide of 15 cc. of solution prepared from 10 cc. of 1 °/₀ egg-albumin (initial* p_H *7·08), and 5 cc. of formaldehyde solution (initial* p_H *7·07).*

Alkali added (cc.)	Mean p_H of blank	Mean p_H undenatured	Mean p_H denatured
0·00	4·87	4·87	4·89
0·20	5·39	—	—
0·30	5·54	4·98	4·97
0·50	5·68	5·07	5·06
0·75	5·91	—	—
0·80	—	5·43	5·41
1·00	6·33	5·66	5·70
1·50	7·49	6·14	6·13

It will be observed that the actual p_H range covered in these titrations with alkali is 4·87 to 6·14, in spite of the fact that the separate solutions of formaldehyde and protein are initially at p_H 7·08. The acidity produced is due to interaction of formaldehyde with ammonium salt as already explained.

Table V. *Corrected titres for undenatured egg-albumin.*

p_H	Titre (cc.)	Blank (cc.)	Corrected titre
4·87	0·00	0·00	0·00
4·98	0·30	0·045	0·255
5·07	0·50	0·080	0·420
5·43	0·80	0·230	0·570
5·66	1·00	0·450	0·550
6·14	1·50	0·890	0·610

Table VI. *Corrected titres for denatured egg-albumin.*

p_H	Titre (cc.)	Blank (cc.)	Corrected titre
4·89	0·00	0·00	0·00
4·97	0·30	0·045	0·255
5·06	0·50	0·075	0·425
5·41	0·80	0·220	0·580
5·70	1·00	0·510	0·490
6·13	1·50	0·885	0·615

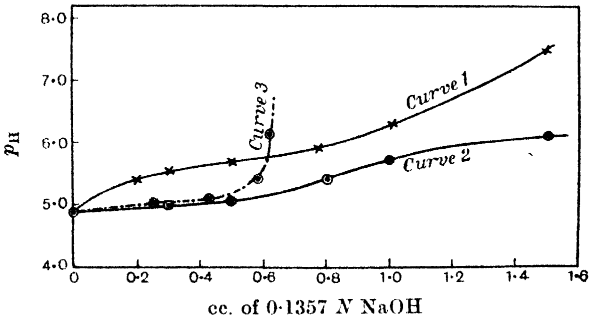

Fig. 2. Titration of egg-albumin with alkali.

Curve 1, titration of solvent alone.
Curve 2, titration of undenatured and denatured albumin.
Curve 3, corrected titre for undenatured and denatured albumin.

DISCUSSION.

Reviewing the above results we see that both in the case of acid and alkali titrations there is no appreciable difference in behaviour between undenatured and denatured egg-albumin. Such small differences as are manifest are attributable to experimental error. The question naturally arises whether the amount of acid or alkali added for titration was sufficient to denature the egg-albumin. In the case of acid titrations it can be quite definitely stated that this was not the case. At a temperature of 25° denaturation only proceeds at an appreciable rate when the p_H of the solution is below 1·75. Thus at p_H 1·72, 1·6 % is denatured in 2 hours, and at p_H 1·02 less than 10 % in 100 minutes. In the above experiments the titration curve becomes approximately asymptotic to the p_H axis when a p_H of 2·75 is reached. Hence there is no chance of appreciable denaturation occurring during the few minutes required for taking the reading.

In the case of titrations with alkali it is unlikely that any denaturation occurred as the p_H only alters from 4·9 to 6·15, in which region the protein is stable until a temperature considerably higher than 25° is reached. However the system here is very complex and it is not definitely known to what extent formaldehyde, formic acid, and the other reaction products present may bring about denaturation, and consequently it is not possible to speak with the same certainty.

Harris [1923, 1] states that he finds no evidence of any increase in the titration figure on denaturation, but gives no indication of how he arrived at such a conclusion. The writer's results agree, however, with this statement. More recently Hendrix and Wilson [1928] performed titration experiments on uncoagulated and heat-coagulated egg-albumin, and found that the uncoagulated protein combined with more acid and alkali than did the coagulated protein. Although their results appear to have been accurately performed it seems probable that their coagulated protein had been subjected to rather more severe treatment than simple denaturation followed by flocculation. In the first place they heated the protein at the isoelectric point, at which p_H hydrolytic scissions are known to accompany the denaturation process; an alteration in p_H will be observed due to this. Secondly they heat it in steam for half an hour, which seems to be an unnecessarily prolonged treatment at a p_H as low as 4·7. And thirdly they allow it to stand overnight before performing the titration experiments, thus allowing possible further decomposition of the protein, with a corresponding alteration in titratability. The writer considers these remarks to be justified by the fact that when Hendrix and Wilson subjected the egg-albumin to still more severe treatment, by washing with alcohol, drying in an oven at 60°, and pulverising, a still smaller ability to combine with acid and alkali was found. It is concluded that Hendrix and Wilson's results do not really bear upon the problem of simple denaturation.

The number of "free" titratable basic and acidic groups.

By the term "free" is meant the free amino- and carboxyl groups only in the protein molecule. From the titration curves it is calculated, assuming the molecular weight of egg-albumin to be 43,000 [Marrack and Hewitt, 1929], that the egg-albumin molecule contains 25·3 groups titratable by acid, and 36·5 titratable by alkali. It follows from the results of Osborne, Jones and Leavenworth [1909] that the following titratable amino-groups are present in egg-albumin:

$$\left.\begin{array}{lll} \text{histidine} & \ldots & 11{\cdot}0 \times 10^{-5}\ \text{mols.} \\ \text{arginine} & \ldots & 28{\cdot}2 \times 10^{-5}\ \text{mols.} \\ \text{lysine} \ldots & \ldots & 25{\cdot}7 \times 10^{-5}\ \text{mols.} \end{array}\right\}\ \text{per g. albumin.}$$

These values are calculated from the number of total nitrogen atoms, on the basis that one half of the total lysine-nitrogen, one third of the histidine-nitrogen, and one quarter of the arginine-nitrogen will be titratable. Hence

1 g. of egg-albumin contains $64{\cdot}9 \times 10^{-5}$ titratable amino-groups, or 27·8 titratable groups per molecule. This agrees reasonably well with the writer's titration value of 25·3 for titration with acid. Incidentally the number obtained by titration is not necessarily a limiting value even for free amino-groups.

Further, from the results of P. S. Lewis [1927] it can be calculated, assuming a molecular weight of 68,000 for haemoglobin [Adair, 1925], that there are 97·6 groups titratable by acid. From results by Van Slyke [1911] on the amount of histidine, arginine, and lysine present it follows that we should expect 99·3 titratable groups to be present. Thus in the case of both proteins we have good agreement between the calculated and "found" number of free groups titratable by acid. Owing to the absence of data with regard to the amounts of the various dicarboxylic acids present in egg-albumin and haemoglobin it has not been possible to compare the number of carboxyl groups with the number of groups titratable by alkali or by acid, the latter being a possibility on the zwitterion basis.

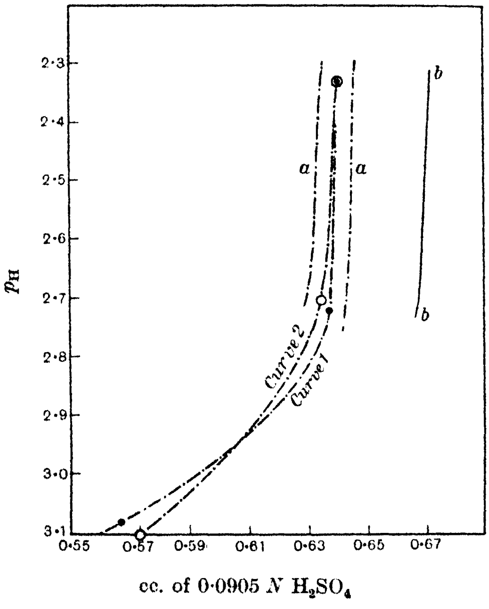

Fig. 3.

Curve 1, titration of undenatured albumin.
Curve 2, titration of denatured albumin.
a, *a*, limits of error. *b*, *b*, position of curve for one extra titratable group.

The question now arises as to whether an increase or diminution of a very small number, say one, amino- or carboxyl group would be detected in the titration experiments in the case of egg-albumin. Let us consider the more accurate series of results, namely those involving titration with acid. The latter portion of the titration curve is plotted on the accompanying graph (Fig. 3) on a large scale, together with the limits of experimental error, and the position of the end-point if one group more were present. From this graph it will be clearly seen that a change of one group titratable by acid would be detected by a perceptible alteration in the titration curve. Hence it can be definitely stated that no change in acid titratability accompanies denaturation.

The possible interpretation of the titration values.

Denaturation may be accompanied by a transition of the protein in part at least from the "ordinary" form into the zwitterion form. Such a hypothesis would at least be in agreement with the ease with which denatured material is flocculated. It is realised of course that zwitterion formation if it occurs is inadequate in itself to explain denaturation. Thus it does not explain the irreversibility of the process, and the high critical increment.

On the basis of the zwitterion theory, titration with acid involves a formation of un-ionised COOH groups thus:

$$—COO^- + H^+ \rightarrow —COOH.$$

Thus titration with acid is titration of $—COO^-$ groups, and in a similar way titration with alkali is titration of charged amino-groups $—NH_3^+$. However, any transition into the zwitterion form on denaturation if such occurs will not be revealed in the titration experiments for the following reason.

Let us suppose for the sake of clarity that prior to denaturation all the amino- and carboxyl groups are present in the "ordinary" un-ionised form at the isoelectric point, and that we have 25 $—NH_2$ groups and 36 —COOH groups. Suppose that on denaturation some number not greater than 25, let us say arbitrarily 5 amino-groups, change over into the $—NH_3^+$ form. In order that zwitterions may be formed this must be accompanied by a change of 5 carboxyl groups into the form $—COO^-$. Hence we now have 20 $—NH_2$ and 5 $—COO^-$ groups titratable by acid. This is a total of 25 as was the case before denaturation, and hence we should not expect to find any difference in the acid titration curves before and after denaturation. In a similar way we have 36 —COOH groups titratable by alkali before denaturation, and 31 —COOH plus 5 $—NH_3^+$ groups titratable afterwards, so that again no difference in titration curves would be expected. This conclusion would hold even if the whole 25 NH_2 groups changed into the zwitterion form together, necessarily, with 25 COOH groups. Obviously this represents the maximum number of zwitterions that could possibly be formed in the case considered. Actually the case of titration by alkali is somewhat complicated by the presence of formaldehyde. The function of the formaldehyde is to combine with the basic group of the amino-acid (see equation (2) p. 167).

Harris [1929] has pointed out that on the old (as opposed to the zwitterion) view the apparent basic dissociation constant remains unchanged on addition of formaldehyde, while the apparent acidic dissociation constant increases, and that this effect is anomalous, as combination with the basic group should affect the basic constant. According to the new (or zwitterion) view the apparent basic constant relates in actuality to the true acidic constant, and *vice versa*. Hence addition of formaldehyde causes a diminution of the true basic constant and leaves the true acidic constant unchanged. Thus the effect of formaldehyde no longer appears anomalous, and Harris cites this as evidence in favour of the zwitterion theory for amino-acids.

In the case of every amino-acid which is present to any extent in the zwitterion form there is an equilibrium between the absolutely un-ionised form and the zwitterion form:

$$NH_2 . R . COOH \rightleftarrows {}^+NH_3 . R . COO^- \qquad \ldots\ldots(1).$$

On addition of formaldehyde the $—NH_2$ group reacts with it, while the ${}^+NH_3$ does not.

$$NH_2 . R . COOH + H . CHO \rightleftarrows CH_2 : N . R . COOH \qquad \ldots\ldots(2).$$

Thus, provided the dissociation constant of the group $CH_2 : N—$ is lower than that of $—NH_2$, the addition of formaldehyde will lower the true basic dissociation constant of the amino-acid. The presence of the equilibrium (2) will naturally decrease the amount of zwitterion present in the solution, by moving the equilibrium (1) from right to left. For a given amount of formaldehyde added there will be a certain proportion of zwitterions, un-ionised amino-acid, and imino-compound. Addition of NaOH to this system will cause two reactions to take place simultaneously:

$$CH_2 : N . R . COOH + NaOH \rightarrow CH_2 : N . R . COO^- + Na^+ + H_2O \ldots\ldots(3),$$

$${}^+NH_3 . R . COO^- + NaOH \rightarrow NH_2 . R . COO^- + Na^+ + H_2O \qquad \ldots\ldots(4).$$

The latter will of course be followed by

$$NH_2 . R . COO^- + H . CHO \rightarrow CH_2 : N . R . COO^- + H_2O \qquad \ldots\ldots(4a),$$

We see that by either of the reactions (3), or (4) and (4*a*) (taken together), we arrive at the same system on addition of a certain amount of alkali. Hence the p_H attained by addition of a given amount of alkali will not be altered by the initial presence of a larger (or smaller) proportion of the zwitterion form. Hence we should not expect any difference in the alkali titration curves even if the denatured protein does exist in the zwitterion form to a greater extent than does the undenatured protein. To demonstrate the actual formation of zwitterions if such occurs in the act of denaturation will require a different type of experimental test.

SUMMARY.

1. It is shown that the titration experiments of P. S. Lewis with haemoglobin do not actually demonstrate that the titratability of undenatured and denatured proteins is the same, owing to the fact that the haemoglobin undergoes denaturation during the titration for all p_H values less than 4 and greater than 10. The present investigation is concerned with crystallised egg-albumin which can be titrated in the undenatured state owing to the relative slowness of denaturation even at p_H 1, and 25°.

2. Titration experiments with acid using the quinhydrone electrode, and with alkali using the glass electrode, have been carried out. It is found that in neither case does heat denaturation affect the number of titratable groups. It is shown that a change of one titratable group (if such change occurred) would be well beyond the limits of experimental error.

3. Using the value 43,000 obtained by Marrack and Hewitt for the molecular weight of egg-albumin, it is calculated that there are at least 25 groups "easily" titratable with acid and 36 similar groups titratable with alkali. The number of free amino-groups found by analysis is 27, which agrees with the writer's acid titration values.

4. It is shown that, if zwitterion formation does accompany denaturation, such a change cannot be detected by any alteration in titratability of the protein.

This investigation was carried out under the direction of Prof. W. C. M. Lewis, F.R.S. The author wishes to acknowledge a grant made by Imperial Chemical Industries, Ltd. to the Department of Physical Chemistry of the University of Liverpool.

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XXII. LACTIC ACID FORMATION IN MUSCLE EXTRACTS.

V. A COMPARISON BETWEEN SOLUBLE STARCH AND GLYCOGEN IN RESPECT OF LACTIC ACID FORMATION AND PHOSPHORIC ESTER ACCUMULATION.

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In a previous paper [Stiven, 1929] glucose and glycogen were used in investigations similar to those described in this paper. It was found in these experiments that, with the phosphate-bicarbonate extracts of cat muscle prepared in this laboratory, glucose differed from glycogen in showing practically no phosphoric ester accumulation while at the same time showing in general more extensive formation of lactic acid at the end of 2 hours' incubation. The experiments reported in this paper have been made to compare soluble starch and glycogen with regard to lactic acid formation and phosphoric ester accumulation.

Results.

In a previous paper [Stiven, 1928] will be found the details of the method of preparing the extract from perfused cat muscle and of the manner of incubation. The "inorganic" phosphorus, which, as estimated by the method of Fiske and Subbarow [1925], includes the phosphorus of phosphagen along with the true inorganic phosphorus, was determined at the intervals shown in the tables. The lactic acid was estimated by the method of Friedemann, Cotonio and Shaffer [1927] at the same intervals. The soluble starch used was supplied by British Drug Houses and yielded on hydrolysis 89 % of its weight of glucose. A correction was made for this in making up the stock 10 % solution to be added to the extract and a similar procedure was adopted with the glycogen. Thus the concentration of soluble starch in the extract was made comparable with that of the glycogen on the basis of the yield of glucose on hydrolysis.

In six of the eight experiments recorded, the yields of lactic acid from soluble starch and glycogen respectively, at the end of 2 hours' incubation, are much alike. In No. 260, starch has given rise to less lactic acid than glycogen

Table I. *Lactic acid formation from glycogen and soluble starch both present to* $0{\cdot}5\ ^{\circ}\!/_{\circ}$.

(Average concentration of lactic acid at start 250 mg. per 100 cc.)

Exp.		Lactic acid formed (mg. per 100 cc.) in 10 min.	20 min.	30 min.	40 min.	50 min.	60 min.	90 min.	120 min.
258	Glycogen	68	126	178	228	275	309	342	357
	Soluble starch	59	116	154	202	250	284	—	356
260	Glycogen	87	127	159	183	207	229	260	260
	Soluble starch	63	108	140	159	171	178	186	190
261	Glycogen	46	101	139	154	175	192	259	301
	Soluble starch	43	72	105	136	173	199	266	302
262	Glycogen	54	90	125	161	194	216	250	252
	Soluble starch	26	50	77	106	135	163	284	304
263	Glycogen	36	72	107	134	150	160	189	220
	Soluble starch	18	37	60	82	103	120	168	219
267	Glycogen	49	75	86	110	126	140	172	200
	Soluble starch	26	53	77	99	120	138	192	218
268	Glycogen	54	73	88	127	143	162	195	216
	Soluble starch	34	54	82	107	131	158	188	194
272	Glycogen	56	98	123	169	208	237	309	331
	Soluble starch	41	72	104	135	167	196	268	336

Table II. *Phosphoric ester accumulation with soluble starch and glycogen both present to* $0{\cdot}5\ ^{\circ}\!/_{\circ}$.

(Average concentration of "inorganic" phosphorus at start 55 mg. per 100 cc.)

Exp.		Decrease of "inorganic" phosphorus (mg. per 100 cc.) below initial concentration at 10 min.	20 min.	30 min.	40 min.	50 min.	60 min.	90 min.	120 min.
258	Glycogen	16	6	8	14	27	24	7	0
	Soluble starch	6	4	4	4	4	4	4	0
260	Glycogen	27	38	32	24	15	5	0	0
	Soluble starch	11	5	0	0	0	0	0	0
261	Glycogen	11	20	44	46	40	31	7	0
	Soluble starch	2	4	6	8	10	10	20	16
262	Glycogen	6	4	3	3	2	1	0	0
	Soluble starch	2	0	0	0	0	0	0	0
263	Glycogen	5	7	9	15	18	33	28	15
	Soluble starch	0	0	0	0	0	0	0	17
267	Glycogen	44	53	47	42	35	30	15	3
	Soluble starch	1	0	0	0	0	0	15	16
268	Glycogen	39	41	32	24	15	7	0	0
	Soluble starch	2	0	0	0	0	6	13	4
272	Glycogen	13	25	32	37	31	22	0	0
	Soluble starch	3	4	6	4	3	3	5	0

but this is reversed in No. 262. By following the lactic acid production during the first hour, it will be seen that in every case during the first 40 to 50 minutes the rate of lactic acid production is less with soluble starch than with glycogen. This more rapid formation of lactic acid from glycogen is associated, as will be seen from Table II, with the more rapid formation of phosphoric ester from glycogen. While it is the case that starch shows ester accumulation, in this respect differing from glucose, yet it never shows this phenomenon to nearly the same extent as glycogen nor does the point of maximum ester accumulation in the case of starch coincide as regards time with that shown by glycogen. In four of the cases, the ester accumulation with starch is delayed until the second hour.

Meyerhof [1926] has investigated the lactic acid production from starch, glycogen, amylopectin, trihexosan and allied compounds and he has found that at the end of 90 minutes' incubation these compounds have yielded practically the same amount of lactic acid. The above experiments are in agreement with Meyerhof's findings as regards lactic acid production at the end of that period of time. Meyerhof suggests, on the basis of an idea of Pringsheim, that this similarity is due to the formation from each of these polysaccharides of a trihexosan. Lohmann [1926] has isolated a triamylose as an intermediate product of the action of muscle-diastase on glycogen. It does not follow, however, that this common intermediate is formed from starch at the same rate as from glycogen. The lower rate of lactic acid formation from starch in the early period of the incubation and the less rapid phosphoric ester formation from starch seem to indicate that the production of intermediates suitable as substrates for the esterifying enzyme takes place more slowly from starch than from glycogen. This great rapidity of ester formation when glycogen is used is one of the outstanding features of this carbohydrate and serves to distinguish it from soluble starch and glucose, which, in the type of extract prepared in this laboratory, resemble glycogen as regards lactic acid production at the end of an incubation of 1–2 hours.

SUMMARY.

Details are given of the results of lactic acid production and phosphoric ester accumulation with glycogen and soluble starch respectively in extracts from the perfused skeletal muscle of eight cats. During the first 40–50 minutes of the incubation, the rate of lactic acid formation and the rate of phosphoric ester accumulation are greater with glycogen than with soluble starch. At the end of 2 hours, however, the lactic acid formation from soluble starch is practically the same as that from glycogen.

In concluding, the author wishes to express his thanks to Prof. E. Waymouth Reid, F.R.S., for his assistance in perfusing the cats.

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XXIII. LACTIC ACID FORMATION IN MUSCLE EXTRACTS.

VI. THE INFLUENCE OF IRRADIATION ON LACTIC ACID FORMATION AND PHOSPHORIC ESTER ACCUMULATION FROM GLYCOGEN.

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The results of previous investigations on the effect of exposure of enzymes to ultra-violet light indicate that the effect is usually destructive. The recent investigations of Kumanomidoh [1928] on diastases, of Uehara [1928] on pepsin and of Hayashi [1928] on lipase, under the direction of Pincussen, all point to that conclusion. With this in mind we have investigated the effect of exposure of muscle extract to a quartz mercury vapour lamp. There is no basis for assuming that in the complex enzyme system of muscle extract the resistance to the destructive influence of ultra-violet light is the same for each individual enzyme. We hoped for that reason that by carefully controlled irradiation of many extracts we might obtain more knowledge about the relationship between phosphoric ester accumulation and lactic acid formation when glycogen is the substrate. The results, which were quite unexpected, are given in the following thirteen experiments.

METHOD.

The cats were killed by coal gas, thereafter perfused and the muscles extracted with a phosphate-bicarbonate solution exactly as described in a previous paper [Stiven, 1928, 1]. The extracts were concentrated to about half the volume by freezing out the excess of water. The whole extract was then centrifuged in a Sharples super-centrifuge to remove debris of muscle fibres and, in particular, droplets of fat. The removal of these latter is very essential because they adhere to the wall of the quartz tube during irradiation. On centrifuging, the fat droplets coalesce and can be removed by filtration through glass wool, although the greater portion of the fat remains behind in the bowl of the centrifuge. This treatment has no effect on the activity of the extract. At this stage, the reaction of the whole extract was adjusted to about p_H 7·0 and divided into two portions. The control unirradiated sample was kept at about 10° while the remainder was being irradiated. For the purpose of irradiating we constructed a grid of clear quartz tubing to fit in front of the window of the lamp box. This window has an area of 27 by 23 cm. The grid was built up of fifteen pieces of quartz tubing, 7·5 cm. apart, each piece 27 cm. long. The tubing had a 5 mm. bore and a wall 0·5 mm. thick. These parallel tubes were connected together by U-shaped pieces of ordinary glass tubing. The extract had thus to flow through the whole length of the quartz tubing, in all 405 cm., while exposed to the lamp. The centre of the grid was 13 cm. from the lamp, which is 8 inches long, taking 3·5 amps. at a burner voltage of 150 volts. The extract was run in from a large funnel surrounded by ice and water and was caught on exit in an enamelled cup, immersed in ice and salt. There were two such receivers, used alternately, so that the flow of the extract was never interrupted. The minimum volume of extract that could be conveniently used under these conditions was 210 cc. These precautions for cooling were essential because by merely keeping the extract, without addition of substrate, for a short time at about 37° changes quite different from those produced by irradiation can be brought about. The extent of the heating may be judged from the fact that if the temperature of the extract before it entered the grid was about 5°, the temperature on leaving was about 17°. As a temperature of 17° was one which we could most easily control, we decided to maintain this throughout the series. If the temperature showed any sign of rising above 17° the rate of flow was accordingly increased. The cooling in the receiver reduced the temperature to about 4°: hence our reason for keeping the unirradiated control at 10° during the period of irradiation. At the end of the irradiation, the p_H of the control was compared with that of the irradiated sample, but there was never any difference. The two samples were then divided up as required and glycogen added to the extent of 0·5 % throughout the series. Details of the method of incubation are given in a previous paper [Stiven, 1928, 1]. It may be pointed out here that the above method is not the same as that employed in Pincussen's investigations. The irradiation and the incubation do not proceed contemporaneously as in the method employed by Pincussen's workers.

The lactic acid was estimated by the method of Friedemann, Cotonio and Shaffer: the phosphagen plus true inorganic phosphorus, referred to here as "inorganic" phosphorus, was determined by the method of Fiske and Subbarow.

RESULTS.

In Table I lactic acid formation from 0·5 % glycogen in the control samples is compared with that in the corresponding irradiated sample. The experiments are arranged in ascending order of intensity of irradiation. By this method, other things being equal, the extent of the irradiation is directly proportional to the time and inversely proportional to the volume of extract in circulation through the grid. Thus irradiation of 250 cc. for 25 minutes should be equivalent

Table I.

		Lactic acid formed (mg. per 100 cc.) in								
	Exp.	10 min.	20 min.	30 min.	40 min.	50 min.	60 min.	90 min.	120 min.	Time of irradiation of 250 cc. (min.)
268	Control	54	73	88	127	143	162	195	216	
	Irradiated	69	96	109	123	136	150	177	207	20
266	Control	17	44	51	57	—	69	—	108	
	Irradiated	52	64	78	89	99	108	—	129	25
267	Control	49	76	86	110	126	140	172	200	
	Irradiated	77	184	198	224	246	262	298	330	25
269	Control	66	92	112	131	150	173	212	248	
	Irradiated	105	169	185	200	217	234	266	291	30
264	Control	42	63	82	100	118	138	159	172	
	Irradiated	77	135	162	190	219	241	277	301	30
270	Control	34	41	57	71	83	88	—	96	
	Irradiated	73	80	89	98	106	113	—	130	30
263	Control	36	72	107	134	150	160	189	220	
	Irradiated	77	113	133	147	157	166	195	202	35
271	Control	26	36	48	67	76	84	86	89	
	Irradiated	72	106	117	129	139	146	164	168	35
273	Control	60	81	98	116	144	165	195	202	
	Irradiated	127	217	294	343	374	388	422	432	45
274	Control	65	125	147	164	179	195	236	251	
	Irradiated	137	277	357	395	427	448	448	448	60
275	Control	70	104	123	137	145	150	154	161	
	Irradiated	17	34	51	82	104	109	128	135	90
276	Control	55	106	152	186	217	239	319	355	
	Irradiated	99	198	275	319	351	355	364	370	95

Table II.

		Decrease of "inorganic" phosphorus (mg. per 100 cc.) in								
	Exp.	10 min.	20 min.	30 min.	40 min.	50 min.	60 min.	90 min.	120 min.	Time of irradiation of 250 cc. (min.)
268	Control	39	41	32	24	15	7	0	0	
	Irradiated	35	42	36	31	25	21	11	1	20
266	Control	27	26	22	17	13	8	0	0	
	Irradiated	30	30	27	25	22	19	1	0	25
267	Control	44	53	48	42	36	30	16	3	
	Irradiated	36	47	40	32	25	18	3	0	25
269	Control	26	52	48	38	33	25	5	0	
	Irradiated	41	56	52	46	41	36	25	13	25
264	Control	9	9	9	7	7	6	3	0	
	Irradiated	35	43	36	29	24	18	5	0	30
270	Control	51	48	44	38	33	25	0	0	
	Irradiated	51	56	53	51	48	44	25	7	30
263	Control	5	7	9	16	18	33	28	14	
	Irradiated	45	54	50	45	40	36	27	19	35
271	Control	38	42	40	34	26	18	0	0	
	Irradiated	28	48	48	46	41	37	8	2	35
273	Control	26	54	50	45	36	27	1	0	
	Irradiated	20	28	23	11	3	0	0	0	45
274	Control	8	32	49	43	37	30	9	0	
	Irradiated	23	27	11	0	0	0	0	0	60
275	Control	33	51	51	44	37	29	5	4	
	Irradiated	1	1	0	0	0	0	0	0	90
276	Control	5	13	23	33	40	32	10	0	
	Irradiated	15	25	18	6	0	0	0	0	95

to irradiation of 210 cc. for 21 minutes. In all but two of the experiments 250 cc. were irradiated. In these two exceptions the volume used was 210 cc., but for convenience of comparison the times in the tables are expressed on the basis of 250 cc.

With regard to the lactic acid formation, it will be seen from Table I that the shortest irradiation produced a slight increase in the rate of lactic acid formation in the first 30 minutes of incubation. As we pass to longer irradiations the difference between the irradiated and the control samples becomes more pronounced, reaching its maximum when the irradiation has been continued for 45 to 60 minutes as in Exps. Nos. 273 and 274. In these two experiments the higher rate of lactic acid formation in the irradiated samples is maintained as long as there is glycogen present. Taking the average rate of lactic acid formation during the time that glycogen is present, as indicated by the iodine test, say during the first 50 minutes, we can see that in No. 273, 100 cc. of the control extract have formed per minute about 2·5 mg. lactic acid as compared with 7·5 for the irradiated sample: in No. 274, these values are respectively 3·6 for the control and 8·5 for the irradiated. The total yields at the end of 2 hours' incubation in the irradiated samples are double those in the controls.

This falling off of the originally high rate of lactic acid formation is due to lack of substrate which in these irradiated samples is being used up at about three times the rate in the controls. Exp. No. 275, with an irradiation period of 90 minutes, shows a decided destruction of the enzyme activity and we have produced the phenomenon referred to at the beginning of the paper.

In No. 276 which appears to have been more resistant to irradiation than No. 275, the slight destructive effect is somewhat overbalanced by a previously produced acceleration so that it resembles, at least as regards lactic acid formation, one of the extracts that has had a much shorter exposure.

The results of the "inorganic" phosphorus estimations in Table II again show that irradiation has had a decided effect. After the shorter irradiations, the extent of the phosphoric ester accumulation is as a rule increased, particularly so if the control sample does not show much accumulation, as in Exps. Nos. 263 and 264. When the irradiation has been continued for 45 to 60 minutes the effect is now one of diminished ester accumulation, as shown in Exps. Nos. 273 and 274; but this decrease is evidently not accompanied by any decrease of the lactic acid formation. On the contrary, these two cases are outstanding by the great increase of the rate of lactic acid formation after irradiation. In Exp. No. 275, which showed a decided inhibition of the lactic acid formation after irradiation, we find that the ester accumulation has been almost completely inhibited. Exp. No. 276 indicates with regard to its phosphorus changes that slightly longer irradiation would have made it comparable to No. 275.

A better idea of the changes is to be obtained by studying the graphs of Figs. 1 and 2, showing the results of Exp. No. 277, in which we first irradiated 350 cc. for 30 minutes, removed 140 cc. and continued the irradiation of the

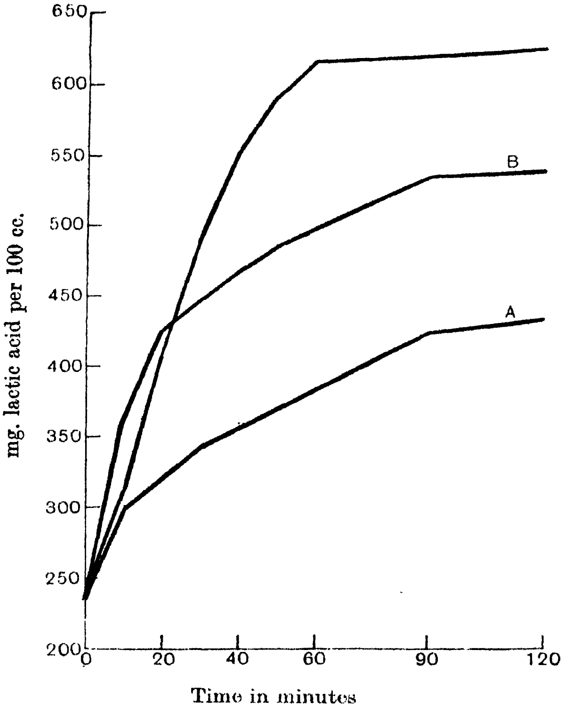

Fig. 1. Exp. No. 277. Lactic acid formation from 0·5 % glycogen.
A. Control. B. After short irradiation. C. After long irradiation.

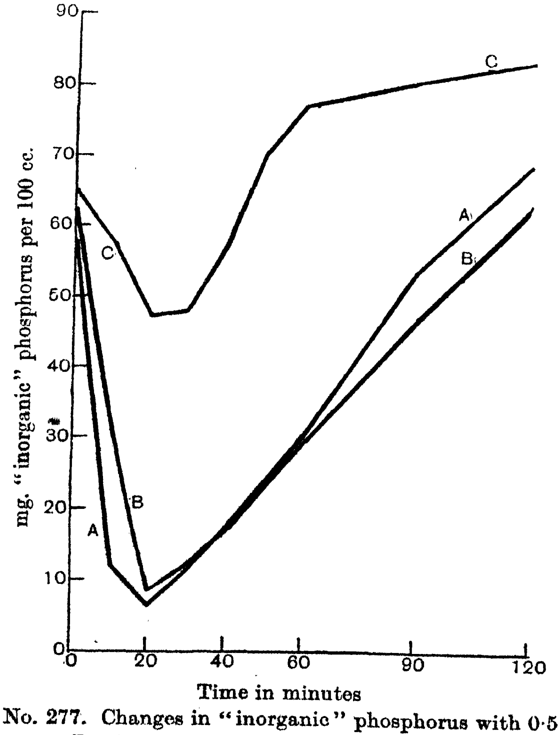

Fig. 2. Exp. No. 277. Changes in "inorganic" phosphorus with 0·5 % glycogen.
A. Control. B. After short irradiation. C. After long irradiation.

remaining 210 cc. for a further 30 minutes. Whilst the short irradiation has produced very little effect on the "inorganic" phosphorus changes, the effect on the lactic acid formation is quite definite. The longer irradiation, whilst reducing the phosphoric ester accumulation, has produced a still more extensive increase in the rate of lactic acid formation.

DISCUSSION.

Jacoby [1928] has noted an increase in the rate of lactic acid formation in hirudinised blood when irradiated during incubation. Beyond this we have failed to find anything in the literature similar to the results given above. One interesting feature of the results, in addition to the fact that irradiation can produce changes resulting in increases of the rate of lactic acid formation from glycogen, is that the highest rates of lactic acid formation are obtained when the extent of the ester accumulation has been reduced. The author has pointed out in previous papers the difficulty of arriving at any decision on the subject of the relationship between ester accumulation and lactic acid formation. In particular, it was possible by adding zymophosphate along with glycogen to increase the extent of the phosphoric ester accumulation, which resulted in a decrease of the lactic acid formation [Stiven, 1928, 2]. The results of this paper make the matter even more complicated because it is shown here that irrespective of increase or decrease of the ester accumulation the lactic acid formation is invariably increased by appropriate irradiation and the highest rates of lactic acid formation are associated with decreased ester accumulation.

It is impossible at this stage to give any explanation of the mechanism of the effect of ultra-violet light on muscle extract. It must be kept in mind that if glucose is used in place of glycogen as the source of lactic acid, the effects are quite different from those described above. Further communications will be made on this subject.

SUMMARY.

In twelve of the thirteen experiments described in this paper it has been found possible to increase the rate of the lactic acid formation from glycogen if muscle extract be irradiated before incubation. In one case the rate of lactic acid formation in the irradiated sample was three times that in the control. Irradiation leads also to alteration of the phosphoric ester accumulation. The shorter irradiations resulted in an increase of the extent of the ester accumulation, the longer irradiations produced a decrease. The highest rates of lactic acid formation coincide with a decrease of the ester accumulation. In only one case was the lactic acid formation decreased after long irradiation, and in this case the ester accumulation was almost completely inhibited.

The author is indebted to Prof. E. Waymouth Reid, F.R.S., for assistance in perfusing the cats.

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XXIV. STRUCTURE AND ENZYME REACTIONS.

VIII. THE PHYSICAL STATE OF THE SORBENT.

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INTRODUCTION.

PREVIOUS research on the influence of structure on enzyme reactions has shown that the sorbent may influence the course of such reactions in two ways: (1) by inactivation of the adsorbed enzyme, and (2) by the spatial separation of the substrate from the enzyme, in those cases in which both undergo adsorption [cf. Przyłecki, 1929].

If we represent the total quantity of enzyme present in a given heterogeneous system by E, that of the adsorbed and free enzyme by E_1 and E_2 respectively, of active and inactivated enzyme by aE and iE respectively, whilst total, adsorbed, and free substrate are represented by S, s_1 and s_2, then the quantity of enzyme-substrate compound formed will be some function of E and S in the absence of a sorbent, and of $(E_1, S) + (E_2, S)$ in its presence. In those cases in which $S = s_2$, the velocity of reaction in the presence of a sorbent will depend upon the degree of activity of E_1 and E_2. Where $aE_1 = aE_2$, it was considered that the velocity of reaction in the presence of a sorbent not affecting the substrate would be the same as in a homogeneous system, whilst where $E_1 = iE_1$ and $E_2 = aE_2$, it would depend solely upon E_2S.

The effect of spatial separation of the enzyme from the substrate is most marked in those cases in which $aE_1 = aE_2$.

Where both enzyme and substrate are adsorbed, the velocity of reaction is the sum of the velocities of the individual reactions taking place between adsorbed and unadsorbed enzyme and substrate in the various phases of the system. The term E_1s_1 is in macroheterogeneous systems negligible, as a result of the difficulty of contact between E_1 and s_1. This view has been confirmed by experiments in which the sorbent was present as a suspension (charcoal) or as a colloidal gel (ovalbumin), whilst experiments carried out conjointly with Dr J. Niedzwiecka [1928] on the influence of ovalbumin sols have indicated that the physical state of the sorbent influences the reaction between E_1 and s_1.

The subject of this paper is the detailed examination of the influence exerted on the velocity of enzymic reactions by changes in the physical state of the sorbent. This problem is extremely interesting in view of the fact that within the cell, according to the recent researches of Spek [1928], the colloids present, which may act as adsorbents, are in a state of flux.

On the whole, biologically important sorbents may be divided into two classes—dispersoid colloids, and those present at liquid-liquid interfaces, such as between drops of fats and plasma. Changes in the former class may consist in changes in the degree of dispersion, which are often associated with passage from sol to gel, or the reverse, and may result in diminution of the adsorbent surface.

The surface tension at the boundaries colloid-solvent often changes with the degree of dispersion of the colloid. As the colloidal micelles increase in size, the concentration of physically adsorbed substances increases. In particular, abrupt changes in systems in which membrane formation takes place, or in which transient surface tension changes occur, may lead to changes in the above relations E_1/E and s_1/S.

Thus, for example, if starch solution be added to a protein solution in which a foam has been formed by agitation, a considerable portion of the starch will undergo adsorption on the protein membrane-water interfaces. At the moment that the foam is broken, and the protein membranes consequently disappear, the adsorbed starch should return into solution. In our experiments this did not, however, take place. The adsorbed starch apparently undergoes some physical alteration, as a result of which, after the resolution of the foam, it separates as a precipitate which settles at the bottom of the vessel; this precipitation is absent from systems which have not been shaken.

The formation of this precipitate leads to changes in the velocity of reaction. The system contains finally s_1, s_2 and s_0 (precipitate), an equilibrium

$$\begin{tikzcd}
s_1 \arrow[rr, shift left] \arrow[dr, shift left] & & s_2 \arrow[ll, shift left] \\
& s_0 \arrow[ul, shift left] \arrow[ur] &
\end{tikzcd}$$

probably existing, and s_0 does not to any considerable extent enter into combination with E_1 or E_2. The velocity of reaction will now consist of the following terms:

$$f_1(E_2s_2) + f_2(E_1s_1) + f_3(E_1s_2) + f_4(E_2s_1) + f_5(E_1s_0) + f_6(E_2s_0).$$

The reaction is thus retarded to an extent commensurate with the magnitude of s_0, and relatively independent of E_1/E.

The existence of such phenomena will be demonstrated on the system shaken, ovalbumin solution-starch solution, in which kinetic experiments show that at least 85 % of s_0 does not enter into the reaction (see p. 186).

The passage of monons (primary particles) into polyons, as well as increase in the size of monons, may lead to the occlusion of originally adsorbed micelles of enzyme or substrate, the quantity of micelles thus occluded being proportional to the ratio original quantity of monons : final quantity of polyons. This phenomenon may be expressed as follows: $E_1 \rightarrow E_1^{\alpha} + E_1^{\beta}$, and

$s_1 \rightarrow s_1^\alpha + s_1^\beta$, the terms designated by α and β denoting respectively free and occluded enzyme or substrate. In those systems in which only S is occluded, the velocity of reaction is represented by the sum of various functions of the following terms: $E_1 s_1^\alpha + E_1 s_1^\beta + E_1 s_2 + E_2 s_1^\alpha + E_2 s_1^\beta + E_2 s_2$. The values of the various terms containing s_1^β depend upon the distance between monons of the same micelle as well as on the dimensions of the particles of the substrate and the extent to which they are adsorbed. The effect of occlusion of the substrate upon the velocity of reaction is as a rule greater than that of occlusion of the enzyme, in view of the generally greater size of the particles of the latter, which renders their entry within the polyon difficult. An example of this is given by the system amylase-occluded glycogen-caseinogen, in which the hydrolytic action of the enzyme is completely inhibited.

If s_1^β is excluded from the reaction, the equilibrium point of reversible reactions will be altered, whilst in the case of irreversible reactions these do not practically proceed to completion, as in the above-cited cases of occluded glycogen and amylase. The occluded substrate can take part in the reaction only as a result of a change in the state of dispersion of the sorbent. Occlusion of the enzyme, however, leads in the above case only to a retardation in the velocity of reaction, but not to its complete inhibition. We may similarly conceive that the formation of membranes may be associated with the incarceration of enzyme or substrate particles in separate cells, and with consequent retardation or partial inhibition of reaction.

It is of importance that substances which normally powerfully elute substrates or enzymes from a given sorbent are unable to do so in those cases in which occlusion has taken place, and this appears to us to be possibly a very potent factor in the regulation of enzymic equilibria *in vivo*.

The occlusion and consequent inactivation of enzymes or substrates depends, apart from the distance separating the monons of a given polyon, upon factors influencing the degree of dispersion of the sorbent. Further, substances provoking elution of the adsorbate before aggregation of the sorbent to a large extent prevent subsequent occlusion.

An example of retardation of reaction due to occlusion of the enzyme alone is given by the system ovalbumin-occluded saccharase-chloroform (W. Giedroyć, unpublished work). In this case, as a result of the selective elutive effect of chloroform upon the substrate alone, the ovalbumin, on coagulation, contains only the ferment.

Diminution of the degree of dispersion of the sorbent is associated with diminution of the Brownian motion of its particles. Since, as has been shown in a previous part of this series, practically no reaction can, as a result of their spatial separation, take place between enzyme and substrate particles adsorbed on one and the same micelle, enzyme and substrate are free to interact only by the collision of different micelles on which they are adsorbed, and the possibility of such collisions is greater the greater the degree of dispersion of the sorbent, and consequently, the greater the Brownian motion of its micelles. This was illustrated in Part II of this series, by the system ovalbumin-amylase-glycogen.

Cessation of Brownian motion may be accompanied by the arrangement in some definite order of the monons combining to form polyons, whereby structure formation commences; this may still further retard enzyme reactions, in two ways:

(*a*) even stirring or shaking cannot facilitate contact of enzyme adsorbed on one micelle with substrate adsorbed on another;

(*b*) quite apart from sorption, the velocities of diffusion of enzyme and substrate are diminished.

In systems containing two liquid phases, such as oil-water, changes in the oil-water interface may take place with the greatest facility. This interface possesses surface energy, which is diminished by the presence of starch or glycogen, which thereby undergo adsorption on the oil surfaces. Increase in the dispersion of the oil will therefore lead to an increase in the proportion of adsorbed glycogen, with a corresponding diminution in its concentration in solution. The reverse does not, however, apply. Coagulation of the drops of oil with a consequent decrease in free surface leads not to a corresponding increase in concentration of dissolved polysaccharide, but to the precipitation of the latter.

The above considerations show that retardation of reaction may be due not only to inactivation of adsorbed enzyme and to spatial separation of simultaneously adsorbed enzyme and substrate; a number of other possibilities may exist in which retardation is due to the physical state of the substrate, whilst the enzyme may remain active and unadsorbed. Thus both the precipitation of the substrate and its occlusion may reduce the velocity of reaction, as well as affect its equilibrium point. These are particular cases of the effect of the state of the substrate on enzyme reactions; this problem will further be considered in the subsequent papers of this series.

EXPERIMENTAL.

All experiments were comparative. Sørensen's phosphate buffers were used to ensure uniform p_{H}, which was 6·2 in the systems containing oil or protein and 6·6 in those with gelatin. Adsorption was measured at room temperature immediately after shaking, whilst precipitation was examined 24 hrs. after commencement of shaking. The kinetics of all systems was examined either in a thermostat at 37° ± 0·5°, or at 25° ± 1°. In all cases protein was removed before determination of glucose.

I. THE SYSTEM OIL-STARCH-AMYLASE.

Adsorption of starch. 30 cc. of 0·5 and 1 % solutions of starch are shaken for 2 hrs. with, in some cases, 5 cc. of olive oil, in 100 cc. Erlenmeyer flasks, the contents of which are then filtered; 10 cc. of the filtrate are hydrolysed at 100° for 4 hrs. with 1·5 cc. of concentrated hydrochloric acid, and reducing sugars determined by Bertrand's method. The results, given in Table I, show that whilst in the absence of oil, 10 cc. of 1 and 0·5 % starch solutions yield respectively 92·5 and 52 mg. of sugar, in its presence only 68·2 and 34·2 mg. are obtained. It follows that 26·3 % of the starch present in 1 %, and 34·2 % of that in 0·5 % solutions have undergone concentration on the oil surfaces; increasing the time of shaking from 2 to 4 hrs. does not appear to affect the values obtained. At the same time, the emulsion is stabilised by the starch, in the absence of which separation of the phases takes place in 30–60 mins., whilst in its presence the emulsion lasts over 24 hrs. As the globules of oil coalesce, the greater part of the adsorbed starch is precipitated, a certain amount remaining, however, in adsorption. The addition of propyl alcohol to 1·4 % or of butyl alcohol to 0·71 % leads to a considerable diminution in the degree of adsorption of starch, which now amounts to 11·8 and 14·7 % respectively (see Table I).

Table I. *Adsorption and elution of starch on oil.*

Mg. glucose in 10 cc. of filtrate, using 30 cc. of 0·5 and 1 % starch, and 5 g. of oil.
Each result is the mean of four determinations.

		Oil							
		No alcohol				With propyl alcohol		With butyl alcohol	
Starch %	No oil	2 hrs. shaking	% sorption	4 hrs. shaking	% sorption		% sorption		% sorption
0·5	52·0 (51·0–53·0)	34·2 (32·6–36·2)	34·2	34·3 (32·4–36·5)	34·0	45·9 (41·4–47·1)	11·8	44·4 (43·3–47·8)	14·7
1·0	92·5 (91·5–93·5)	68·2 (66·1–70·4)	26·2	—	—	—	—	—	—

Adsorption of amylase. Parallel experiments to the above were carried out using 0·5 % amylase (Merck's) in 1 % sodium chloride solutions in place of starch. To 20 cc. of the filtrates after shaking, 20 cc. portions of 1 % starch solution were added and the flasks left at 37° for 4 hrs. after which reducing sugars were determined as before. The results, given in Table II, show that in the presence of oil 20 cc. of filtrate produced 67·5 mg. of sugar, and in its absence 71 mg., a difference of 4·9 %. It follows that amylase is not to any great extent concentrated on the oil-water interface.

Table II. *Sorption of amylase on oil.*

Mg. glucose in 40 cc. solution, containing 20 cc. of 1 % starch, after 4 hrs. at 37°.

No. of exps.	Without oil	With oil	% sorption
4	71·0 (70·0–72·5)	67·5 (65·5–69·3)	4·9

Kinetics of the system oil-water-amylase.

(*a*) ***Emulsion.*** A number of flasks containing 30 cc. of 0·5 % starch solution and 10 cc. of 1·5 % amylase solution were shaken for 5 hrs. at 25°, and the reducing sugar content of the filtrates compared with that of a similarly treated series of flasks to which 5 cc. of olive oil had been added. The results, given in Table III, show that in the first case 47·9 mg. of sugar were found in 10 cc. of the filtrate, whilst in the presence of oil 45·4 mg. were found, a difference of 5·2 %.

Table III. *Influence of oil on kinetics of hydrolysis of starch by amylase.*

Mg. glucose in 20 cc. of filtrate, using 0·5 % starch solution.

	No oil		Oil			
No. of exps.	With shaking	Without shaking	Amylase added before shaking	% inhibition	Amylase added after shaking	% inhibition
4	47·9	48·0	45·4	5·2	37·8	20·1
	(47·2–48·5)	(47·0–49·1)	(45·2–46·6)		(37·6–38·9)	

This inhibition may be due either to simultaneous adsorption of substrate and enzyme or to the inactivation of the latter. In the first case, substitution in the equation $ES = E_1s_1 + E_1s_2 + E_2s_1 + E_2s_2$ gives

$$0{\cdot}05 \times 0{\cdot}34 + 0{\cdot}05 \times 0{\cdot}66 + 0{\cdot}95 \times 0{\cdot}34 + 0{\cdot}95 \times 0{\cdot}66 = 1{\cdot}7\ \%\ ES,$$

i.e. the maximal retardation of reaction due to simultaneous adsorption is 1·7 %; in reality the value obtained would be even lower in view of the fact that the system is shaken, whereby the possibility of contact occurring between substrate and enzyme particles in adsorption on different globules of oil is increased. The observed inhibition of reaction must therefore be due to inactivation of adsorbed enzyme. It is in this connection of interest that Truszkowski [1928] found that amylase adsorbed on lipoid surfaces is thereby inactivated, and, as this is the first case published of inactivation due to physical adsorption, it is our intention further to investigate this phenomenon.

(*b*) *The precipitate.* The above experiments were repeated, with the difference that amylase was added 24 hrs. later, when a considerable portion of the starch had been precipitated. The results, given in Table III, show that in the absence of oil 47·3 mg. of sugar were formed, and in its presence 37·8 mg., a difference of 20·2 %. This retardation is due preponderatingly to precipitation and consequent exclusion from reaction of starch as s_0. Since about 30 % of the starch is, in emulsions, present at the interface, it follows that only 67·7 % of the adsorbed substrate undergoes precipitation.

II. THE SYSTEM OVALBUMIN-AMYLASE-STARCH.

Adsorption and elution of starch. To one series of flasks, containing 30 cc. of 0·5 % starch, 5 cc. of 8 % Merck's ovalbumin solution are added, and to another 5 cc. of water; all flasks are then shaken for 2 hrs., their contents filtered, and the reducing sugar content of the filtrates determined after hydrolysis with hydrochloric acid, as before. The results, given in Table IV, show that, in the presence of ovalbumin, 62·7 % less starch is present in the filtrates than in its absence. Substantially the same results are obtained if the flasks are shaken 4 hrs. instead of 2. This phenomenon appears to be due to the adsorption of starch on the protein membranes present in the foam formed by shaking, and these membranes are retained by the filter-paper. Starch adsorbed on dissolved protein passes together with the latter through the filter-paper, so that determinations made on the filtrates apply to both free starch and to that adsorbed on the colloidal sol.

The addition of alcohols in the same concentrations as those used with the corresponding system containing olive oil similarly diminishes the amount of adsorption. Thus, as is shown in Table IV, the proportion of adsorbed starch is diminished by 67·5 % by propyl alcohol and 27·0 % by butyl alcohol.

If the system is left for 24 hrs. after shaking, a precipitate of starch forms, as in the presence of oil, and the amount of this precipitate is considerably smaller in the presence of alcohols. No precipitate forms if the system is not shaken.

Table IV. *Sorption and elution of starch in shaken ovalbumin solutions.*

Mg. glucose after hydrolysis of filtrate of the system 30 cc. 0·5 % starch + 5 cc. 4 % ovalbumin.

		Ovalbumin							
		Shaken 2 hrs.						Shaken 4 hrs.	
No. of exps.	No ovalbumin	No alcohol	% sorption	Propyl alcohol	% sorption	Butyl alcohol	% sorption	No alcohol	% sorption
4	45·2 (44·9–45·5)	14·7 (14·4–16·0)	67·5	37·3 (35·1–38·6)	17·5	33·0 (31·9–35·2)	27·0	12·5 (12·2–13·5)	72·2

Adsorption of amylase. To one series of flasks containing 20 cc. of 1 % amylase 5 cc. of 10 % ovalbumin solution are added, and to another series 5 cc. of water. All are then shaken for 2 hrs., filtered, and to 10 cc. of each filtrate 20 cc. of 0·5 % starch solution are added, and the amylase allowed to act for 4 hrs. at 37°. The results given in Table V show that about 29·6 % of the amylase present is adsorbed on the protein membranes.

Table V. *Sorption of amylase in shaken ovalbumin sols.*

Mg. glucose 4 hrs. after addition of 10 cc. of filtrate from 20 cc. of 1 % amylase solution and 5 cc. of water or of 10 % ovalbumin to 20 cc. of 0·5 % starch solution.

No. of exps.	Without ovalbumin	With ovalbumin	% sorption
4	42·2 (40·8–43·4)	29·7 (27·0–32·4)	29·6

Kinetics.

(*a*) *In constantly shaken systems.* Assuming that on the average 67 % of the starch and 29·6 % of the amylase are in adsorption on the protein membranes present in the foam, then, even neglecting possible retardation of reaction due to simultaneous adsorption of enzyme and substrate on the particles of the sol, $E_1 s_1 = 19{\cdot}8\ \%\ ES$, *i.e.* the retardation of reaction should amount to about 20 %. A comparison of the reducing sugar contents of the filtrates of flasks shaken for 2 hrs. at 25°, and containing 30 cc. of 0·5 % starch solution and 10 cc. of 1 % amylase solution, with the addition of 5 cc. of 10 % ovalbumin solution or of water, indicates (see Table VI) that hydrolysis is barely 3·5 % less in the presence of ovalbumin. It follows that retardation due to the term $E_1 s_1$ is only 17·5 % of the theoretical maximum. In this case, therefore, the presence of a sorbent of both enzyme and substrate does not involve the absence of reaction between E_1 and s_1.

(*b*) *After cessation of shaking.* The preceding experiment was repeated, with the difference that shaking took place at 5°, after which the flasks were left for 2 hrs. at 25°, when the reducing sugar content of the filtrates was determined. These modifications should not lead to any great changes in the ratios E_1/E and s_1/S, yet, as is shown in Table VI, the percentage inhibition is now 12·7 %, as compared with 3·5 % in the preceding experiment, *i.e.* the reaction between E_1 and s_1 is inhibited to the extent of 60·3 %. In no case was precipitation of starch observed. The retardation of reaction should therefore be ascribed to the absence of agitation, which in the previous experiment facilitated contact of enzyme and substrate particles adsorbed on different micelles.

Table VI. *Influence of ovalbumin on kinetics of hydrolysis of starch.*

Mg. glucose in 20 cc. of filtrates of systems containing 30 cc. of 0·5 % starch, 10 cc. of 1 % amylase, and 5 cc. of water or of 10 % ovalbumin solution.

		Ovalbumin					
No. of exps.	No ovalbumin	Shaken 2 hrs.	% inhibition	Shaken 2 hrs. at 5°, then left 2 hrs. at 25°	% inhibition	Shaken 2 hrs., then left 24 hrs., amylase added, and left 2 hrs. at 25°	% inhibition
4	43·3 (42·8–43·6)	41·8 (41·6–42·1)	3·5	37·8 (35·7–38·3)	12·7	21·6 (20·4–22·1)	50·0

(*c*) *24 hrs. after cessation of shaking.* Exp. *a* is repeated with the difference that amylase is added 24 hrs. after cessation of shaking, *i.e.* after the formation of a precipitate of starch. We see from Table VI that the reaction is now inhibited to an extent of 50–54 %. Since 55–60 % of the starch has been precipitated, 10 % of this must have entered into reaction.

Table VII. *Kinetics of enzymic hydrolysis in the presence of ovalbumin at different degrees of coagulation.*

Duration of heating of ovalbumin secs.	Mg. glucose	% inhibition
0	39·0 (37·9–40·1)	0
15	37·8 (35·6–39·2)	3·1
60	34·0 (32·8–35·0)	12·9
90	32·2 (30·7–33·1)	17·4
300	30·9 (30·1–32·0)	20·7

(*d*) *The same system containing ovalbumin in various stages of coagulation.* A number of flasks containing 20 cc. of 1 % ovalbumin solution were heated with constant shaking for 0, 15, 30, 60 and 90 secs., and 5 mins. in a water-bath at 100°. The flasks were then cooled and to each 15 cc. of 1 % starch solution and 10 cc. of 1 % amylase solution were added. The flasks were then shaken for 2 hrs. and left at 25° for 3 hrs., after which the volume of solution was in all cases made up to 50 cc., and the reducing sugar content determined. The results, given in Table VII, indicate that inhibition of reaction between E_1 and s_1 rises from 0 in the presence of unheated protein to 100 % where the latter had been heated 5 mins.

III. THE SYSTEM CASEINOGEN-GLYCOGEN-AMYLASE.

10 cc. of 2 % glycogen were added to 100 cc. of milk, the whole was shaken for 2 hrs., after which hydrochloric or acetic acid was added to p_H 3·0–4·2, and the solution left for 6 hrs. for precipitation of caseinogen, after which N sodium hydroxide solution was added to p_H 5·0–5·2. A second series of flasks was submitted to the same operations, with the difference that the 10 cc. of glycogen solution were added after alkalisation. All flasks were now shaken for 2 hrs., after which 25 cc. of 1 % malt amylase solution were added, and the flasks left at 25°. After 7, 30 and 90 days glycogen was determined in the whole solution, in the filtrate and in the residues.

The results, given in Table VIII, show that not even a trace of glycogen was present in any of the filtrates, and glycogen was also absent from the residues of those flasks to which it had been added after precipitation of caseinogen, as a result of the reaction $s_1 \rightarrow s_2$, and of the interaction of s_1 with E_2 and of s_2 with E.

Table VIII. *Inhibition of glycogenolysis due to occlusion.*

Each result is the mean of four experiments.

Mg. glycogen found in the systems

	Curdled milk to which glycogen and amylase were added			Glycogen and amylase added to milk which is then curdled								
	No alcohol		2 % Butyl alcohol ppt.	Without alcohol			Alcohol added after curdling of milk			Alcohol added before curdling of milk		
Duration of exp. days	Whole system	Ppt.		Whole system	Ppt.	Filtrate	Whole system	Ppt.	Filtrate	Whole system	Ppt.	Filtrate
0	200·0	176·8	69·4	202·1	179·6	23·0	201·7	174·0	23·2	201·0	84·6	118·2
7	39·7	36·1	23·8	143·5	141·0	3·0	140·9	138·7	0	70·7	63·7	0·7
30	0	0	0	138·1	137·4	0	137·0	136·8	0	62·2	61·2	0
60	0	0	0	137·2	137·1	0	136·8	136·3	0	62·4	60·2	0

In those flasks to which glycogen had been added before precipitation, on the other hand, it was present in the residue in all cases, and to an equal extent after 90 days as after 30 days.

In one experiment, for example, 89·4 % of the glycogen present was adsorbed on the caseinogen. After the action of amylase, 68 % of the glycogen remained unhydrolysed, whence it follows that 76·3 % of the adsorbed glycogen was protected from hydrolysis by occlusion.

Action of alcohols on the above system. Repetition of the above experiments, with the addition of propyl alcohol to 2 % before addition of amylase, shows that where glycogen is added after precipitation of caseinogen considerable elution takes place, whilst this is almost entirely absent from those systems to which glycogen had been added before precipitation, and similarly, the velocity of hydrolysis of glycogen is accelerated in the former case by the addition of propyl alcohol but not at all in the latter case.

Entirely different results are obtained if the propyl alcohol be added at the same time as the glycogen. In this case, the proportion of occluded glycogen is greatly diminished, so that after 60 days we find 62·4 mg. per 100 cc. instead of 137·1 mg. as in the preceding experiment.

IV. THE SYSTEM GELATIN-AMYLASE-STARCH.

To a number of flasks containing 10 cc. of 0, 0·9, 2·1, and 3·9 % gelatin, 10 cc. of 0·6 % starch and 10 cc. of 0·9 % amylase were added, and the flasks left for 5 hrs. at 37° and at 25°, after which reducing sugar was determined. The results, given in Table IX, show that 0·3 % gelatin retards reaction by 9·3 % at 37° and by 9·9 % at 24°, 0·7 % gelatin by 17·5 % at 37° and by 21·2 % at 24°, whilst 1·3 % gelatin produces a retardation of 41 % at 37° and of 56·7 % at 24°. The inhibitive action of gelatin thus increases with concentration and with temperature, the relative effect of the latter factor being greater at higher than at lower concentrations of gelatin. The percentage inhibition due to a temperature difference of 13° is 6·5 % with 0·3 % gelatin, 21 % with 0·7 % gelatin, and 38 % with 1·3 % gelatin.

Table IX. *Kinetics of enzymic hydrolysis of starch in presence of gelatin.*

No. of exps.	Conc. of gelatin %	Mg. glucose in 20 cc. of solution after 2 hrs.				Percentage excess of inhibition at 25° over that at 37°
		at 37°	% inhibition	at 25°	% inhibition	
4	0	37·8 (37·6–38·0)	0	28·3 (28·0–28·7)	0	—
4	0·3	34·3 (34·0–34·6)	9·3	25·5 (25·2–25·8)	9·9	6·5
4	0·7	31·2 (30·9–31·5)	17·5	22·3 (22·0–22·6)	21·2	21·5
4	1·3	22·3 (22·0–22·6)	41·0	12·3 (12·1–12·7)	56·7	38·0

SUMMARY.

1. An examination of the system oil-starch-amylase yielded the following results.

Starch undergoes adsorption at the water-oil interface to an extent of 26·3 % from 1 %, and 34·2 % from 0·5 % solutions, in the presence of 5 g. of oil.

The presence of 1·4 % propyl alcohol, or of 0·71 % butyl alcohol, reduces adsorption to about one-half.

About 68 % of the adsorbed starch is deposited as a precipitate from emulsions of oil in starch solution on the resolution of the emulsion.

The adsorption of amylase on oil is very small, not exceeding 5 % under the experimental conditions.

The adsorbed amylase appears thereby to be inactivated and the precipitated starch does not react with amylase.

2. An examination of the system soluble ovalbumin-starch-amylase leads to the following conclusions.

Starch undergoes adsorption to about 63 % on the protein membranes present in the foam formed when ovalbumin solutions are shaken.

The addition of alcohols as in (1) leads to elution of starch.

A portion of the adsorbed starch is deposited as a precipitate on the resolution of the foam.

Amylase is adsorbed on the protein membranes to an extent of about 30 % under the experimental conditions.

In systems containing ovalbumin, starch, and amylase, the velocity of hydrolysis is only 3·5 % less than in the absence of protein if the system is constantly shaken; without shaking, retardation amounts to 12·7 %, whilst when amylase is added 24 hrs. after shaking it amounts to 52 %.

The degree of inhibition due to ovalbumin is proportional to the extent to which it is coagulated.

3. The system caseinogen-glycogen-amylase has been studied, with the following results.

When caseinogen is coagulated in the presence of glycogen, the latter is occluded within the precipitate, and is thereby protected from the action of amylase in the medium, only 24 % of the adsorbed glycogen being hydrolysed.

The occluded glycogen is not eluted by alcohols.

4. A study of the system gelatin-starch-amylase shows that the inhibitive effect on the hydrolysis of starch due to the presence of gelatin increases with concentration of the latter, and is greater at 25° than at 37°.

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XXV. THE STEREOCHEMICAL SPECIFICITY OF ESTERASES.

I. THE AFFINITY OF LIVER ESTERASES FOR OPTICALLY ACTIVE ALCOHOLS.

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Dakin [1904, 1905] first showed that lipases do not hydrolyse the two components of a racemic mixture with equal velocity. He found that liver esterase hydrolysed *d*-ethyl mandelate preferentially when allowed to act on the racemic mixture. Little further work was carried out on the subject until Willstätter and Memmen [1924] and Willstätter, Haurowitz and Memmen [1924] performed the corresponding experiments with pancreatic and gastric lipase. All these observations were confined to the behaviour of the enzyme to the racemic mixture. Rona and Ammon [1927] first investigated the behaviour of the enzyme to the pure optically active forms, and found that liver esterase actually hydrolysed the pure laevo-form with greater velocity than the dextro-form, although selecting the latter from a solution of the mixture. The explanation of this phenomenon was given by Willstätter, Kühn and Bamann [1928]. It was shown that the results were quite consonant with the theory of Michaelis and Menten [1913]. Briefly, the course of hydrolysis depends on the characteristic constants of the two stages of the reaction, which may be represented:

$$\text{Enzyme} + \text{Substrate} \rightleftharpoons \text{Enzyme-substrate-complex} \qquad \text{(I)}$$

and

$$\text{Enzyme-substrate-complex} \rightleftharpoons \text{Enzyme} + \text{Products} \qquad \text{(II)}.$$

All the results may be explained on the single assumption that these constants may be different for the two optical isomers.

In the case of a single substrate, if the substrate concentration is sufficient to saturate the enzyme, the velocity, which is proportional to the concentration of enzyme-substrate-complex, is determined solely by the second constant, but if the enzyme is partitioned between two substrates, the relative velocities of hydrolysis are principally determined by the constants of the first equation, since these determine the relative concentration of the two enzyme-substrate-complexes. Such is the case of a racemic mixture. Mathematically the conditions may be expressed as follows. If K_D, K_L be the constants of equation (I) (affinity constants or Michaelis constants), k_D, k_L the constants of equation (II), c_D, c_L the concentrations of the two enzyme-substrate-complexes, v_D, v_L the velocities of hydrolysis, A the initial amount of each substrate, y and x the amounts hydrolysed at any time, and ϵ the enzyme concentration, then

$$\frac{v_D}{v_L} = \frac{k_D c_D}{k_L c_L} = \frac{k_D K_L}{k_L K_D} \cdot \frac{\epsilon(A-y)}{\epsilon(A-x)} = K \cdot \frac{A-y}{A-x}, \text{ where } K = \frac{k_D K_L}{k_L K_D}.$$

But

$$v_D = \frac{dy}{dt} \text{ and } v_L = \frac{dx}{dt} \text{ so that } \frac{dy}{dt} \Big/ \frac{dx}{dt} = K \cdot \frac{A-y}{A-x},$$

$$\therefore \int \frac{dy}{A-y} = K \int \frac{dx}{A-x} \text{ or } K = \frac{\ln A - \ln(A-y)}{\ln A - \ln(A-x)}.$$

Weber and Ammon [1929] were able to show that the value of K obtained from observations on the racemate was equal to that obtained by multiplying the values of the constants measured separately, thus proving the correctness of the explanation.

It is obvious that experiments on the racemate alone will not give any information on the values of either the affinity constant or the breakdown constant of either form, but only a figure derived from all four. Thus many recorded observations of the "total specificity" of a particular lipase towards a racemic substrate are of little use for presenting a simple comparison between a series of related compounds.

It is now recognised however that enzymes are capable of forming reversible compounds of the Michaelis type with other substances than their substrates, certainly with the products of the reaction and probably with certain other types of quite unrelated compounds. In a system consisting of the enzyme, substrate and a third substance, the extent of formation of these compounds can be easily measured by observing the extent to which the hydrolysis is inhibited, hence the affinity of the enzyme for the substance can be readily measured. If the enzyme-inhibitor-complex is a compound of the Michaelis type, the two isomers of an optically active substance should show a difference in affinity comparable with the difference between the isomers of a substrate.

One of us [Murray, 1929] has already shown that secondary alcohols such as phenylmethylcarbinol inhibit lipase hydrolysis in this way. Such compounds possess the necessary asymmetry, and can be obtained in optically active forms. The three alcohols selected for the first investigation all had a methyl group attached to the carbinol on one side, the other group being in one case aliphatic, viz. *n*-hexyl, in one case aromatic, viz. phenyl, and in the third case mixed, viz. β-phenylethyl.

EXPERIMENTAL METHOD.

The choice of method lay between the gasometric and the direct titrational. The strict constancy of p_{H} gives to the former method a greater accuracy, but experiment showed that for the purpose sufficient accuracy was obtained by the method of direct titration in a buffer-free fluid [cf. Bamann, 1929; Bamann and Schmeller, 1929].

The experiments were performed in test-tubes with a total initial volume of 8 cc. The tubes contained 3 drops of a suitable indicator (bromothymol blue) and whenever the solution became acid to the indicator it was brought back by a drop of $N/100$ NaOH added from a micro-burette which delivered about 40 drops per cc. The error of dilution during titration was found to be negligible. The maximum velocity of hydrolysis was regulated by the amount of enzyme added so that about 30 drops of NaOH solution were required during a half-hour period. All titrations were made at room temperature, and the velocities in a given series were always comparative to one or more control tubes (no inhibitor) in the same series. Usually 10 or 12 tubes were examined together for 30 minutes.

Preparation of material.

Preparation of enzyme. The enzyme extracts were made from preparations of liver, dried by acetone alone, or acetone and ether. The extracting agent was dilute ammonia. $N/40$ was suitable for sheep-liver extraction, but for rabbit-liver $N/10$ was found to be better. After standing for 3 to 4 hours with occasional shaking at about 20°, the residue was centrifuged off, and the solution acidified with acetic acid to precipitate the inactive proteins. The enzyme remained in solution after centrifuging, and, when neutralised with ammonia, was kept in this solution for use. One gram of dried sheep liver extracted with 30 cc. ammonia gives a very active preparation of enzyme, of which 1/20 cc. is sufficient for each tube in this method. Rabbit-liver preparations are less active and contain more buffering matter, largely phosphate, which renders the colour change less easy to follow. A preliminary extraction of the powder with distilled water removes much of this. It has been shown by Willstätter, Bamann and Waldschmidt-Graser [1927] and Rona and Itelsohn-Schechter [1928] that the degree of purification does not affect the stereochemical specificity, so that the above type of preparation is quite suitable.

Preparation of the optically active alcohols. The alcohols were resolved by the method of Pickard and Kenyon [1911]. The method consists of converting them into acid phthalates which are then crystallised with an optically active base. We should like to express our indebtedness to Dr Kenyon who personally gave us the samples of the two forms of methylphenylcarbinol and methyl-β-phenylethylcarbinol.

Choice of substrate and optimal conditions.

Ethyl butyrate proved to be the most suitable substrate. The maximum velocity of hydrolysis with this substrate and sheep-liver esterase is reached at a concentration of about $0{\cdot}005\,M$. With ethyl propionate and this enzyme the maximum velocity is much smaller and is not reached until the concentration is twice as great. Ethyl acetate is very slowly hydrolysed and the maximum is not reached at a concentration of ten times this figure, indicating a much lower affinity between the enzyme and the shorter chain fatty acid esters (Michaelis constant much larger). It is interesting to note that the value of the Michaelis constant is determined far more by the nature of the acid constituent than by the alcohol—thus *n*-butyl acetate was found to behave very similarly to ethyl acetate.

When the concentration necessary for maximum velocity is exceeded, the velocity falls again. Probably an excess of substrate favours the formation of a slowly hydrolysable complex with the enzyme. The curve relating velocity to substrate concentration is shown in Fig. 1, which also shows the effect of a small quantity of inhibitor on the concentration for maximum velocity. The competition of the inhibitor with the substrate for the active enzyme surface delays the inhibitory power of excess substrate.

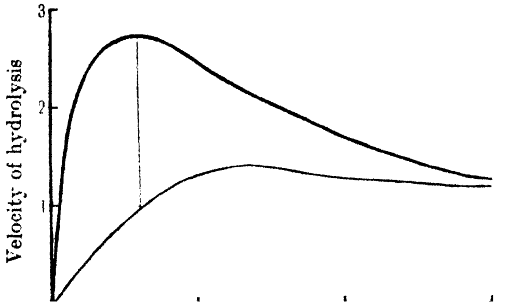

Fig. 1. Effect of 0·00079 *M* methyl-*n*-hexylcarbinol on optimal substrate concentration. Ordinates represent equivalents $\times 10^{-6}$ acid liberated in 10 min.

In all experiments the substrate concentration was that at which the maximum velocity is obtained in the absence of inhibitor. The degree of inhibition is indicated by the vertical drop to the lower curve in Fig. 1.

All the experiments unless otherwise stated were carried out at p_H about 7·0, the indicator used being bromothymol blue.

*Experiments with methyl-*n*-hexylcarbinol.*

The stock solutions of ethyl butyrate and ethyl propionate contained 8 g. per litre, so that when 1 cc. was present in the 8 cc. total fluid the concentration was 1 mg. per cc. For ethyl butyrate, 0·6 cc. was used in each tube, and for ethyl propionate, 1·2 cc., when working with sheep-liver enzyme.

Stock suspensions of the three forms of the sparingly soluble methyl-*n*-hexylcarbinol contained 0·2 cc. (0·165 g.) in 100 cc. Varying amounts of this were added to the substrate and made up to volume, the p_H being adjusted if necessary. The final concentrations of inhibitor were low enough to give complete solution. Enzyme was added to all the tubes and readings commenced. A typical result is given in the accompanying table, and the complete results are conveyed by Fig. 2, which shows the inhibition of the hydrolysis of ethyl butyrate, and Fig. 3, which gives the corresponding curves for ethyl propionate. The inhibition of the latter is much greater, corresponding with the fact that it has a much lower affinity for the enzyme.

Experiment. Inhibition of hydrolysis of ethyl butyrate by the three forms of methyl-*n*-hexylcarbinol. 0·6 cc. ethyl butyrate in all tubes.

	Dextro-						Laevo-						Racemic					
Cc. suspension carbinol ...	0·0	0·15	0·3	0·6	0·9	1·2	0·0	0·15	0·3	0·6	0·9	1·2	0·0	0·15	0·3	0·6	0·9	1·2
Drops $N/100$ NaOH in 10 min. intervals	12	10	8	6	5	4	12	5	3	2	1	1	11	6	4	2	2	1
	12	9	8	6	5	4	12	5	4	2	1	1	11	7	5	3	2	2
	12	10	8	6	6	5	11	6	4	2	2	1	11	6	5	3	2	2
Percentage inhibition		19	33	50	57	64		54	68	83	89	91		42	58	76	82	85

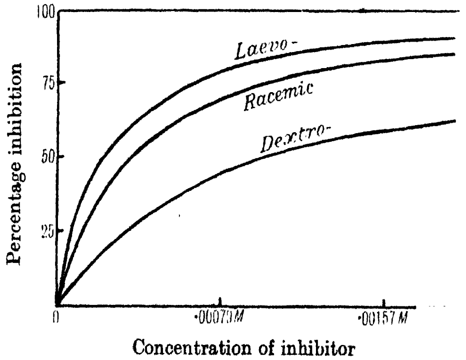

Fig. 2. Hydrolysis of ethyl butyrate inhibited by methyl-*n*-hexylcarbinol (concentration of substrate = 0·00517 *M*).

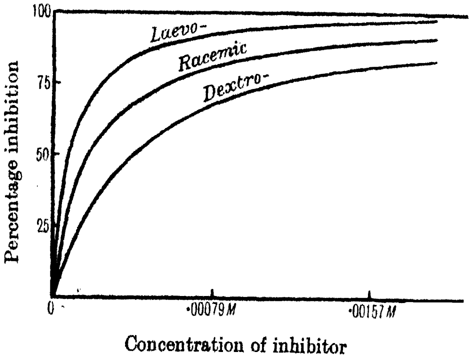

Fig. 3. Hydrolysis of ethyl propionate inhibited by methyl-*n*-hexylcarbinol (concentration of substrate = 0·01176 *M*).

The racemic inhibitor behaves as would be theoretically expected for a mixture of two inhibitors, namely, the concentration required to give 50 % inhibition (equal partition of the enzyme) is given by $2/C = 1/C_D + 1/C_L$, where C_D and C_L are the concentrations of the optically active forms similarly required.

Experiments with methylphenyl- and methyl-β-phenylethyl-carbinols.

Figs. 4 and 5 show the inhibitions given by the two optically active forms of methylphenylcarbinol and methyl-β-phenylethylcarbinol respectively. It will be seen that the general phenomenon of difference between the two forms of the alcohol occurs again, and to a similar extent; that is to say, the laevo-form inhibits from four to five times more powerfully than the dextro-form. The molar concentrations of these alcohols required to give the same degree of inhibition as was given by the aliphatic alcohol, methyl-*n*-hexylcarbinol, differ considerably, the requisite concentration of methylphenylcarbinol being nine to ten times, and that of methyl-β-phenylethylcarbinol about four times greater.

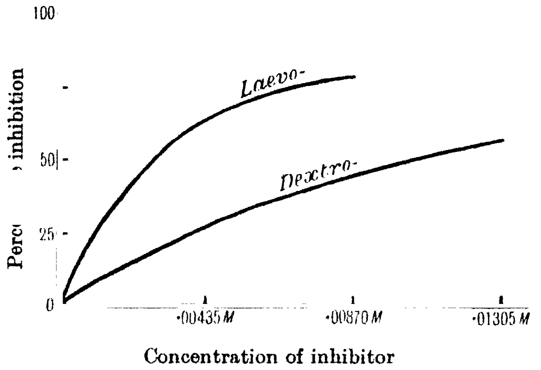

Fig. 4. Hydrolysis of ethyl butyrate inhibited by methylphenylcarbinol.

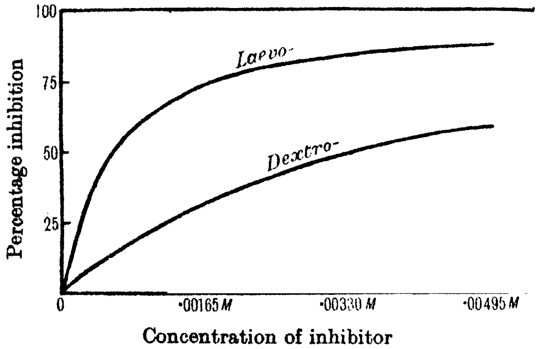

Fig. 5. Hydrolysis of ethyl butyrate inhibited by methyl-β-phenylethylcarbinol.

*Experiments with esters of methyl-*n*-hexylcarbinol.*

The acid phthalates of methyl-*n*-hexylcarbinol were prepared as intermediates in the resolution of the alcohol, and as they were found not to be hydrolysed by the enzyme, their inhibitory properties were tested. The surprising result was obtained that the dextro-form inhibited more than the laevo-form as the following table shows:

	Molar concentration of inhibitor				
	0·0001	0·0002	0·00045	0·0009	0·0018
Percentage inhibition by dextro-form	16	33	49	60	75
Percentage inhibition by laevo-form	13	27	40	51	64

The difference is not so great as with the alcohols, but is well marked. No explanation of this phenomenon is suggested at the present time.

In view of this unexpected phenomenon, the acetates of the three forms were studied. Like the phthalates, they were not hydrolysed by the enzyme. The dextro-acetate was given to us by Dr Kenyon, the laevo- and racemic forms were prepared by ourselves. In this case the result was that all three forms gave nearly the same curve (Fig. 6). This curve differs however from all the others investigated in that it becomes flat at about 70 % inhibition, instead of continuing toward total inhibition. It is possible that this is due to the limit of solubility of the substances, but this view is rendered unlikely by an experiment in which ethyl propionate was used as the substrate. The curve of inhibition is again much steeper with this substrate, and flattens at about 85 % inhibition, but at a concentration where the curve for ethyl butyrate is still rising.

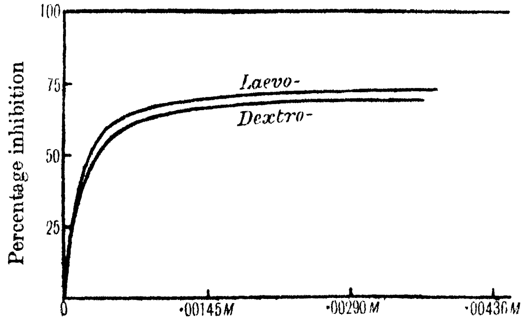

Fig. 6. Hydrolysis of ethyl butyrate inhibited by β-octyl acetate.

Comparison of sheep-liver and rabbit-liver esterases.

When rabbit-liver esterase is used in a similar manner to that described with sheep-liver esterase, the optimum concentration of substrate is higher [cf. Bamann and Schmeller, 1929] being about 1·5 mg. ethyl butyrate instead of 0·6, and the Michaelis constant is correspondingly larger. The inhibitory power of the two isomers of methyl-*n*-hexylcarbinol was found to be very nearly the same for both forms, the laevo-form inhibiting perhaps slightly more than the dextro-form. This was somewhat unexpected since Bamann [1929] found the enzyme to differentiate quite markedly between the two forms of ethyl mandelate, but the Michaelis constant was only determined for one form.

Discussion.

The fact that the two optical isomers differ in the extent to which they inhibit hydrolysis tends to dispose of the objection that the inhibition is merely physical in nature. The similarity of the relation between the forms of the three alcohols further points to a chemical basis for the phenomenon. An asymmetry of the combining group of the enzyme itself probably raises the affinity for one configurational series and lowers it for the antipodal series. It may prove possible to relate this selectivity with that observed in the esters of the hydroxy-acid series. Of the esters, either ethyl or methyl, of mandelic acid, sheep-liver esterase has the greater affinity for the dextro-form. It is known, however [Freudenberg, Brauns and Siegel, 1923], that dextro-mandelic acid is configurationally related to the laevo-series, so that the same grouping in the enzyme structure may be the deciding factor of both phenomena.

Although the combination almost certainly occurs at the polar carbinol group, the remainder of the molecule plays an important part in the process, and appears to determine the measure of the affinity. The length of the non-polar hydrocarbon chain is an important factor, thus the phenyl group is very weak in comparison with the aliphatic six carbon chain, while the introduction of two $—CH_2—$ groups, between the phenyl and the polar group greatly increases the affinity. Preliminary experiments (not recorded in the text) show that one $—CH_2—$ group, as in methylbenzylcarbinol, is as effective as two, while ethyl-*n*-hexylcarbinol appears to be more active than the methyl compound. Further work will be carried out on the effect of substituted and non-substituted hydrocarbon chains of varying length.

Summary.

1. The relative affinities of pairs of optically active secondary alcohols for liver esterases have been studied by observing the extent to which their presence inhibits the hydrolysis of ethyl butyrate or ethyl propionate.

2. The laevo-forms of methyl-*n*-hexylcarbinol, methylphenylcarbinol and methyl-β-phenylethylcarbinol inhibit sheep-liver esterase about four to five times more strongly than the dextro-forms, showing that their affinity is much greater.

3. With rabbit-liver esterase the inhibition caused by both dextro- and laevo-alcohols is practically the same, showing that any difference of affinity is very small.

4. The anomalous behaviour of esters of the alcohols is described.

5. The theoretical implications of the phenomena are discussed.

Our thanks are due to Dr J. Kenyon of Battersea Polytechnic, for supplying the compounds mentioned and some others. It is a pleasure to acknowledge the constant interest of Sir F. G. Hopkins and Mr J. B. S. Haldane in this investigation.

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XXVI. THE VITAMIN B CONTENT OF GRASS SEEDS IN RELATIONSHIP TO MANURES.

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(*Received December 12th, 1929.*)

SINCE carrying out this work we have noted that Colonel McCarrison in 1924 published in the *British Medical Journal* his results on the effect of "artificial" and also cattle manure on the nutritional value of certain grasses. We have also found that C. H. Hunt at Ohio Agricultural Experiment Station in December 1927 performed certain experiments on the effect of what we now call artificial manures on certain wheat plots. We were unaware of this work until a few weeks ago, but we note that our work confirms McCarrison's.

This research was undertaken because one of us (M.J.R.) had noticed that pigs which were fed on home grown and home ground barley and wheat always did much better than those pigs which were fed on purchased barley and wheat, and that certain cattle did better on certain fields. It was decided to find out whether this was due to the lack of lime or of other mineral constituents of the land. The results of this investigation were not satisfactory. It was then decided to try the effect of artificial manure versus dung. In this experiment pigs' excreta mixed with straw were used. These pigs were fed on a diet consisting of 10 % of a proprietary product (containing meat meal, rye and wheat embryo which is high in its vitamin B content, bone meal and cod-liver oil), 40 % of barley meal, and 50 % of middlings.

Two areas of land were pegged out in the same field, on the same side so that both had the same amount of sunlight. The drainage was equal from both areas. This field had previously been an arable one and had grown cabbages in the fifth year before this experiment was carried out, and in the fourth, potatoes. It was then ploughed up and sown with seeds which were made up to contain a small quantity of the different clovers. These seeds were sown with a nurse crop of wheat in 1927, the wheat having been sown in the previous year, 1926. In 1927 the seeds were cut at the time when the wheat was reaped. The field was lightly fed in the autumn of 1927. In 1928 it was cut for hay and grass. In the autumn of 1928 one portion of the land was dunged at the rate of 20 loads of dung to the acre, which is the usual dressing used by the farmer. The other portion was manured with artificial manure, 20 cwt. basic slag plus 3 cwt. kainite to the acre, and in the spring of 1929, 1 cwt. of sulphate of ammonia was applied as a top dressing. In July 1929, these areas were cut by hand and were gathered as soon as dried and placed in separate barns. There was undoubtedly a bigger growth in the dunged area, but not so weighty as that in the artificially manured area. There was more clover in the artificially manured patch. This is what would be expected as the result of manuring with the basic slag. The analysis of the grass seeds showed little difference:

	Protein	Moisture	Fibre	Ash	Phosphorus
	%	%	%	%	%
"Dung" grass seed	12·7	5·8	19·4	9·0	0·44
"Artificial" grass seed	11·7	6·0	21·6	10·5	0·33

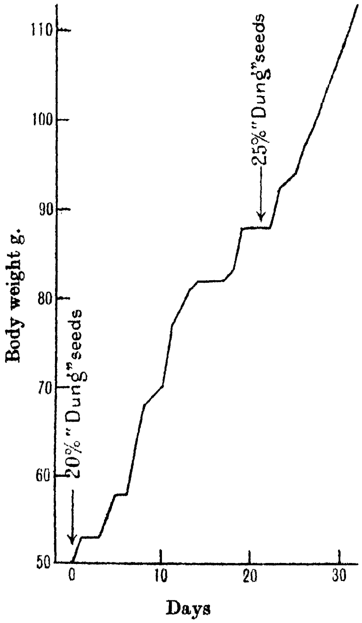

Fig. 1. Rat ηω (♂).

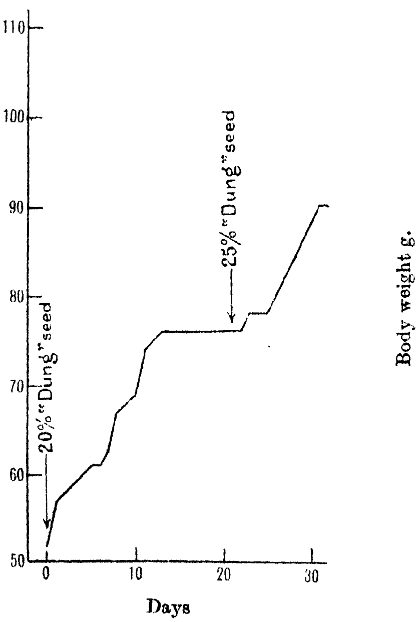

Fig. 2. Rat ηω (♂).

It was evident that grass seeds with such a large fibre percentage were not an ideal diet for the rat, but we decided to test their vitamin B content.

The University College vitamin B deficiency diet of Prof. Drummond was used—rice starch, caseinogen, salt mixture and cod-liver oil. To this were added varying percentages of the grass seeds. Two groups of experiments were carried out, preventive and curative.

In the preventive test, the rats were divided into two lots; one lot was put on deficiency diet to which was added 20 % of the "dung" seed, the other on deficiency diet with 20 % of the "artificial" seed. After 21 days the percentage of seeds was increased to 25 for a further 11 days. The rats on the "dung" seed showed good growth or a slightly subnormal growth. Figs. 1 and 2 are examples of these two types. The rats on the "artificial"

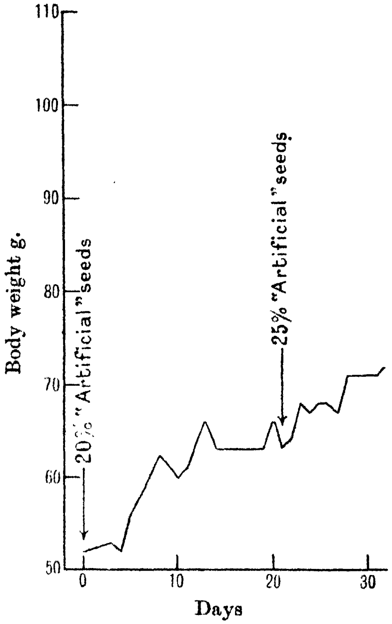

Fig. 3. Rat $\delta\gamma$ (♂).

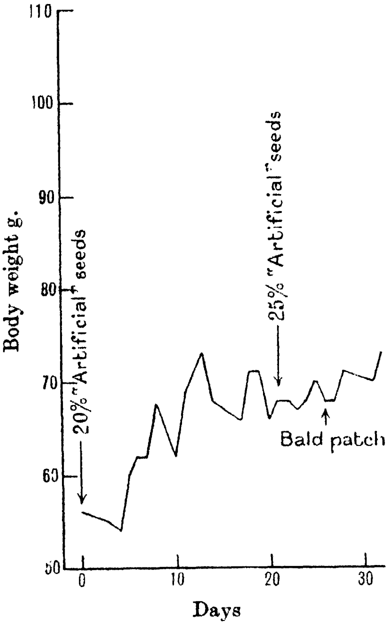

Fig. 4. Rat $\delta\pi$ (♂).

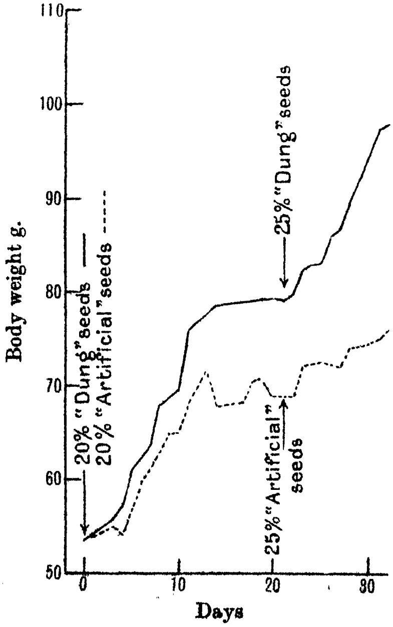

Fig. 5. Rats ηs and δs (♂).

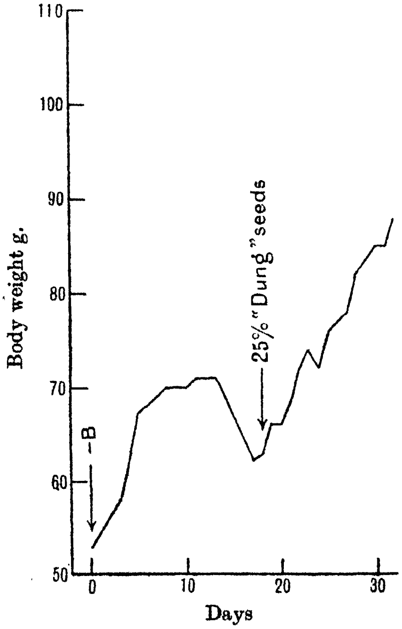

Fig. 6. Rat $\beta\gamma$ (♂).

seeds all grew very poorly, not one giving normal growth. Figs. 3 and 4 illustrate two of these rats. The average growths of the two sets of rats are shown in Fig. 5, the continuous line representing the "dung" seed rats, the broken line the "artificial" seed ones. It can be seen that the former have gained nearly twice as much as the latter, 45 as against 23. The rats on the "artificial" seed were in a poor condition; in some the hair was falling out.

In the curative test rats were put on a vitamin B deficiency diet for 18 days, when all were losing weight. Half of them then had 25 % of "dung" seeds added to their diet and immediately resumed a normal growth curve (Figs. 6, 7, 7 *a*). The other half had 25 % of "artificial" seeds added to their diet. Their condition did not improve but got steadily worse, so that after 3 days it appeared that some of the animals would not live more than a few hours. Their condition was one of typical vitamin B deficiency; they were wasted, hunched and shedding hair. At the suggestion of Prof. Drummond who saw the animals at this time the diet was changed from "artificial" to "dung" seeds. The immediate result was that the rats rapidly recovered, and now showed a normal growth curve (Figs. 8, 9, 9 *a*).

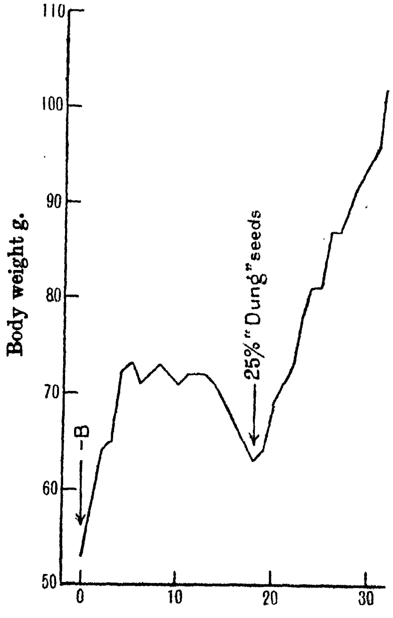

Fig. 7. Rat $\beta\omega$ (♂).

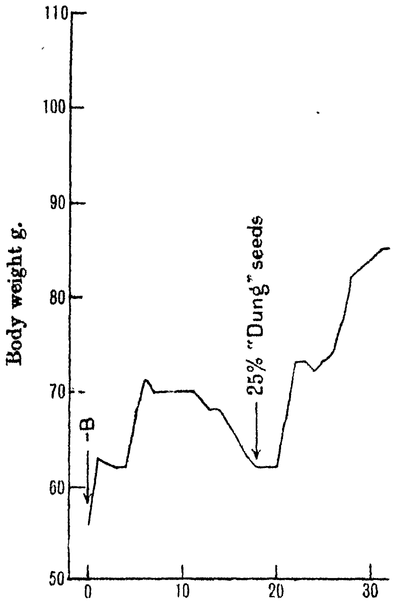

Fig. 7 *a*. Rat $\beta\pi$ (♂).

It would seem that a plant may absorb vitamin B from the land, and that the vitamin B content of any food may be dependent upon the amount of this vitamin in the land. Possibly the view that vitamin B is manufactured entirely in the plant and stored in the embryo is incorrect, and our suggestion would account for the variations in the results of tests carried out by different

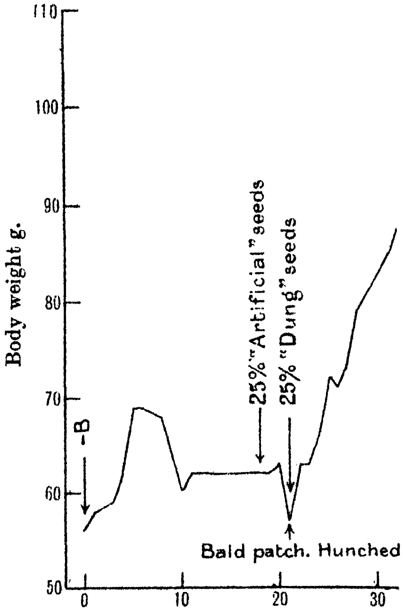

Fig. 8. Rat *aω* (♂).

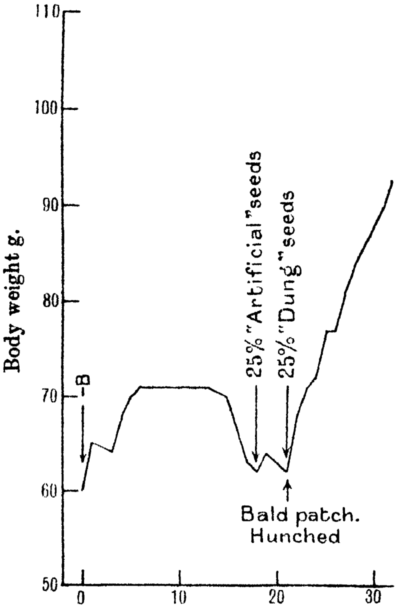

Fig. 9. Rat *aγ* (♂).

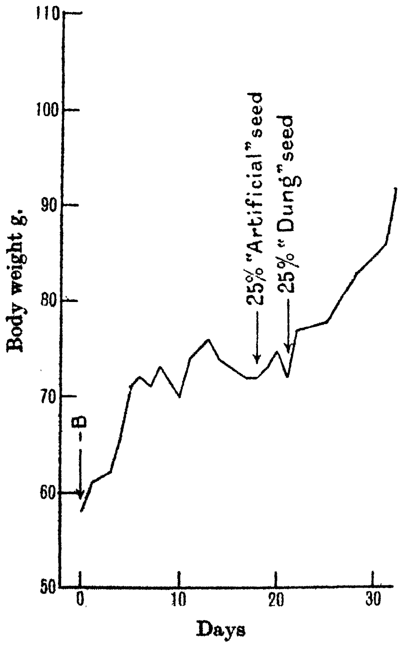

Fig. 9 *a*. Rat *aπ* (♂).

workers. One worker states that in his experimental work a certain food contains vitamin B; another worker using the same type of food differs entirely in his findings, and we consider that the food that is being tested is not the only point which is of importance. If these results are correct, they have a considerable bearing upon agriculture and nutrition.

Further research along these lines is being carried out and the faeces of pigs fed on the above experimental diet are being tested for the presence of vitamin B.

(*Note added January 27th, 1930.*) Since sending this paper for publication, dung from pigs has been tested for its vitamin B content. The pigs were fed on the diet described at the beginning of the paper. An extract was made from the dung with slightly acidified 50 % alcohol. This was concentrated by distilling *in vacuo*, and the concentrate fed to rats that were losing weight on vitamin B deficiency diet. Good growth was promptly begun, showing that there is a considerable quantity of vitamin B in the dung.

XXVII. NOTE UPON THE SULPHUR LINKAGE IN WOOL.

By CLAUDE RIMINGTON.

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(*Received December 7th, 1929.*)

Although the greater part of the sulphur present in wool-keratin can be accounted for as cystine [see Rimington, 1929], the actual state of combination of this amino-acid is still a matter of considerable uncertainty. Thus, if cystine were in simple polypeptide linkage in the protein, it would be expected that suitable treatment of the wool fibre would reveal the presence of free disulphide (—S.S—) or sulphydryl (—SH) groups. That this expectation is not realised will be clear from the experiments recorded here. It seems necessary to conclude that the cystine-sulphur groups in wool are involved in some complex ring system or otherwise obscured so that they cannot react as does free cystine.

Experimental.

A sample of 50's crossbred wool was degreased in warm benzene and then washed thoroughly in many changes of distilled water. Portions of about 0·05 g. were weighed out and subjected to various treatments recorded in Table I. Unless otherwise stated the duration of each test was 24 hours, the tubes being stoppered and left in a dark cupboard. After washing, the lock of wool was divided and tested for free —S.S— and —SH groups in the way recommended by Walker [1924, 1925] using 10 cc. of saturated ammonium sulphate as suspending fluid, and also for general damage by the Pauly reagent. It has been shown by Burgess [1928] and by Burgess and Rimington [1929] that undamaged wool remains uncoloured when immersed in the Pauly reagent, whereas fibres which have suffered mechanical or chemical injury are stained a brownish-red colour proportional to the extent of the injury.

The results are given in Table I, one or more plus signs being used to indicate the intensity of a positive reaction.

An examination of these results warrants the following statements.

(1) Treatment to which free cystine responds readily has little effect upon the sulphur groups in wool. (Compare the effect of reducing agents.)

(2) Considerable alteration of, or "damage" to, the fibre may take place, as indicated by the intensity of the Pauly stain, without any parallel increase occurring in the nitroprusside —S.S— or —SH reactions. If, as is generally

Table I.

Treatment	Duration	—S.S—	—SH	Pauly reaction	Remarks
Control	—	–	–	+ (tips only)	—
Boiling water	20 mins.	–	–	+ (patchy)	No change in appearance
,,	45 ,,	–	–	+ + (patchy)	Yellowish. Characteristic smell appears
,,	1 hr.	–	–	+ + +	Brownish. Pauly stains most intense on discoloured portions
,,	3 hrs.	+ (faint)	–	+ + +	Pauly stain general
Absolute alcohol	24 ,,	±	–	+ (tips only)	—
Formalin 5 %	18 ,,	–	–	±	—
Saturated H_2S solution	18 ,,	+ +	–	–	—
Ultra-violet radiation	80 ,,	+ +	–	+ + + +	Pauly stain general
ENZYMES:					
3·3 % trypsin (p_H 8·0)	48 ,,	+ +	–	+ + +	Pauly stain general
2·02 % pepsin (p_H 1·2)	48 ,,	±	–	+	—
0·1 *N* NaOH alone	24 ,,	+ +	±	+ +	—
BACTERIA:					
B. subtilis (saline)	5 days	–	–	+ +	Growth poor
B. subtilis (broth)	5 ,,	+ (tips only)	–	+ + +	Pauly stain general
B. mesentericus ruber (saline)	5 ,,	–	–	+	—
B. mesentericus ruber (broth)	5 ,,	±	–	+ + +	Pauly stain intense in places
REDUCING AGENTS:					
Sodium sulphite 1 %	18 hrs.	.	+ (tips)	.	—
,,	66 ,,	±	±	+ (tips)	—
Sodium sulphite 4 %	18 ,,	.	+ (tips)	.	—
,,	66 ,,	+ (tips)	+ (tips)	+ +	Pauly stain fairly general
Sodium sulphite 1 % in 0·1 *N* sodium hydroxide	18 ,,	.	+ + +	.	—
,, ,,	66 ,,	+ +	+ + +	+ + + +	Pauly stain strongest at tip
Sodium hydrosulphite 1 %	18 ,,	.	±	.	—
,,	66 ,,	–	–	+ (tips)	Fibres have a white, bleached appearance
Sodium hydrosulphite 4 %	18 ,,	.	±	.	—
,,	66 ,,	–	±	+ (tips)	—
Sodium hydrosulphite 1 % in 0·1 *N* sodium hydroxide	18 ,,	.	+ + +	.	—
,,	66 ,,	+ +	+ + +	+ + +	Fibres very yellow; staining patchy
5 % suspension of calcium oxide in 1 % sodium sulphite	18 ,,	.	+ +	.	—
,, ,,	66 ,,	+ + +	+ + +	+ + + +	Fibres have a soapy feel

CYSTINE SOLUTION (0·3 % IN WATER PLUS SUFFICIENT HCl):

10 cc. of solution used, to which the following reagents were added to produce solutions of the concentrations indicated.

Sodium sulphite 1 %	18 hrs.	.	+ + +	.	—
,, 4 %	18 ,,	.	+ +	.	—
Sodium hydrosulphite 1 %	18 ,,	.	+ + +	.	—
,, 4 %	18 ,,	.	+	.	—
5 % suspension of calcium oxide in 1 % sodium sulphite	18 ,,	Faint pink changing immediately to yellow			

supposed, the behaviour of the wool fibre towards the Pauly reagent is conditioned solely by the state of the epidermal scales, acting as an impenetrable sheath unless mechanically or chemically disrupted, then it would be expected that also in the case of the nitroprusside reaction, where damage was extensive, staining of the cortex would occur. That this is not so seems to show that the failure of the nitroprusside reaction can only be interpreted as indicating the absence of —S.S— and —SH groups.

(3) With the exception of exposure to ultra-violet radiation, only one treatment can be recorded—the action of alkaline solutions—which has been found to have any marked effect upon the sulphur groups of wool. Ultra-violet radiation causes the appearance of —S.S— groups only, whereas after immersion in alkalis the fibre shows positive reactions for both —S.S— and —SH.

DISCUSSION.

Whilst so little is known concerning the structure of proteins, it is impossible to offer a precise explanation of the results recorded in this paper. The keratins, especially, are a group which exhibit many characteristics not met with in typical proteins. The stability and chemical inertness typical of their class have for various reasons been ascribed to the high proportion of sulphur which they contain. The present results show that the sulphur groupings are much less reactive than might have been expected when the lability is recalled of such compounds as cystine peptides and anhydrides.

It would appear that in wool the cystine molecules are bound in some manner involving the sulphur groups and thereby depriving them of reactive freedom. Undenatured egg-albumin presents a somewhat similar case, giving no nitroprusside reaction; on denaturation, however, by whatever means this is accomplished, a strong —SH reaction develops [Harris, 1923]. Blood-serum, normally non-reactive, develops —S.S— groups on denaturation.

Further work is in progress directed towards the elucidation of the sulphur linkage in wool.

SUMMARY.

1. The action of a variety of reagents upon wool has been studied and the conclusion drawn that the sulphur groups of wool are less simple than those of free cystine.

2. Free —S.S— or —SH groups are not present in undamaged wool and can only be induced by the action of alkalis or of ultra-violet radiation.

The author wishes to thank Mr A. T. King of this Association for his interest in these studies.

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XXVIII. A QUANTITATIVE STUDY OF SUCCINIC ACID IN MUSCLE.

III. GLUTAMIC AND ASPARTIC ACIDS AS PRECURSORS.

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(*Received December 30th, 1929.*)

INTRODUCTION.

THAT formation of succinic, malic and fumaric acids takes place in minced muscle from some unknown precursor has been shown in a previous paper [Needham, 1927]. It was also shown that increased formation of these acids followed under anaerobic conditions upon addition of glutamic and aspartic acids to the mass [Moyle, 1924]. In the present work the action of muscle tissue on these two amino-acids and their possible rôle as precursors of succinic acid in the muscle have been investigated.

The production of one molecule of succinic acid from one molecule of aspartic or glutamic acid must involve the removal of nitrogen in some form. It very soon became clear that this form is not ammonia, and further experiments failed to show any increase in urea, uric acid or amide-nitrogen in the muscle samples during the increased production of succinic acid. Indeed, the amino-nitrogen content of these samples estimated by Van Slyke's method was found to be simply the sum of the amino-nitrogen content of the controls plus the amino-nitrogen of the added glutamic or aspartic acid. If one assumes that the same reactions are taking place in oxygen as under anaerobic conditions, but to a greater extent owing to removal of oxidisable reaction products, it might be expected that in oxygen it would be easier to detect some accumulation of nitrogenous non-oxidisable end-products. Estimations of the oxygen uptake in a Barcroft apparatus showed that, with added glutamic or aspartic acid, the increased oxygen uptake was sufficient to account for complete oxidation to carbon dioxide, water and ammonia of from one-half to the whole of the added amino-acid. Nevertheless, no change in the soluble nitrogen fractions could be found, with the exception of a very slight setting free of ammonia. Two possible explanations of these unexpected results were next considered.

In the first place, the increased oxygen uptake consequent upon adding these two amino-acids occurs (in the case of pigeon muscle) to any extent only with unwashed muscle, which shows a very high respiration of its own. Washing the muscle with distilled water reduces the oxygen uptake of the control to about one-tenth of the original value, but it reduces still more the excess oxygen uptake in the samples containing amino-acid. The possibility therefore arises that the glutamic and aspartic acids are acting simply as stimulants, bringing about increased oxidation of other substances. These two amino-acids themselves have no specific dynamic action in the body, but it was thought that, if small amounts of glycine or alanine were formed from them, these might stimulate an increase in carbohydrate metabolism in the muscle; or possibly that traces of ammonia split off by increasing cell permeability might bring about an increase in oxidation. Quantitative experiments, however, lent no support to this view, all the other amino-acids tried, and ammonium salts in different concentrations, having no stimulatory, but rather an inhibitory effect on muscle respiration. It is also difficult to suppose that the succinic acid production following addition of these acids is stimulated from some other source. Further, by direct estimation of the free aspartic acid it was possible to show that the amount present had undergone diminution, although the amino-nitrogen of the sample remained constant.

In the second place, it is possible that the amino-group of the glutamic or aspartic acid may combine with some carbon chain in the muscle, succinic acid and a new α-amino-acid thus being formed. This possibility is further considered later.

FORMATION OF SUCCINIC, MALIC AND FUMARIC ACIDS FROM GLUTAMIC AND ASPARTIC ACIDS.

The muscle was always used as rapidly as possible after the death of the animal. In the case of beef, muscle removed from the neck of the bullock immediately after slaughtering was brought at once to the laboratory in a cooled pan; a long cylindrical muscle could then be dissected out with little injury, and this, after cooling to about 5°, was minced. The pigeons were killed by bleeding, and were then placed breast downwards on ice for some minutes before dissecting off the breast muscles. The muscles were placed in beakers surrounded by ice for about 15 minutes and minced when their temperature had fallen to about 5°. The frog muscles were dissected off and cooled before mincing.

The minced muscle was weighed out into equal samples (20–50 g. being used for each), and each sample was suspended in 2 volumes of 1·4 % $Na_2HPO_4, 2H_2O$, in a Büchner flask. To certain of the flasks the aspartic or glutamic acid, or a mixture of the two, was then added (the amino-acids having been neutralised with sodium hydroxide); an equal volume of water was added to the controls. The flasks were closed with rubber stoppers, and were all evacuated for several minutes; hydrogen was then introduced. The evacuation and filling with hydrogen was repeated twice more. When beef or pigeon muscle was used, the flasks were kept about 2–3½ hours at 37°; when frog muscle was used, they were kept at room temperature for about 26 hours. In many of the experiments the vessels, instruments, etc. were all sterilised by autoclaving; it made no difference to the results whether this precaution was taken or not.

The results are set out in Table I.

Table I.

No. of exp.	Material	Acid added g. per 100 g. muscle	Succinic acid mg. per 100 g.	Total acids as mg. succinic acid per 100 g.	Malic acid mg. per 100 g.	Extra acid formed mg. per 100 g.	Averages		
							Acids added	Extra succinic acid	Extra total acids
S1	Beef	0·4 $\mathrm{H\bar{A}}$ + 0·4 $\mathrm{H\bar{G}}$	83·4	—	—	67·8			
		Control	15·6						
S2	Beef	1·3 $\mathrm{H\bar{G}}$	52·8	—	—	28·8			
			45·6	—	—	21·6			
		1·3 HA	43·0	—	—	19·0			
		Control	24·0						
S3	Beef	0·6 $\mathrm{H\bar{G}}$ + 0·6 $\mathrm{H\bar{A}}$	146		—	121			
			130	—	—	105			
			134	—	—	109			
		Control	26						
			23						
S4	Beef	0·47 $\mathrm{H\bar{G}}$ + 0·6 $\mathrm{H\bar{A}}$	54·4	—	—	37·0	0·49 $\mathrm{H\bar{G}}$ + 0·53 $\mathrm{H\bar{A}}$	71·9	
		1·4 $\mathrm{H\bar{G}}$	43·2	—	—	25·8	1·35 $\mathrm{H\bar{G}}$	25·5	
		Control	17·4				1·3 $\mathrm{H\bar{A}}$	21·6	
S5	Beef	0·7 $\mathrm{H\bar{A}}$ + 0·7 $\mathrm{H\bar{G}}$	—	126	—	110·6			
		Control		15·4					
S6	Pigeon	1·0 $\mathrm{H\bar{G}}$ + 1·0 $\mathrm{H\bar{A}}$	243	—	—	214·8			
		Control	28·2						
S7	Pigeon	2 $\mathrm{H\bar{G}}$	—	85·2	—	37·3			
(see N 1)		1 $\mathrm{H\bar{G}}$ + 0·88 $\mathrm{H\bar{A}}$	—	146·0 (too low)	—	98·1 (too low)			
		Control		47·9					
S8	Pigeon	1·32 HA	—	136·9	—	890			
(see N 8)		0·75 $\mathrm{H\bar{G}}$ + 0·66 $\mathrm{H\bar{A}}$	—	228·2	—	170·3			
S9	Pigeon	0·5 $\mathrm{H\bar{G}}$ + 0·5 $\mathrm{H\bar{A}}$	—	289·0	46	241·1	0·62 $\mathrm{H\bar{G}}$ + 0·58 $\mathrm{H\bar{A}}$	—	205·7
		1·0 $\mathrm{H\bar{G}}$	—	114·6	0	66·7	1·5 $\mathrm{H\bar{G}}$	—	52·0
		1·0 $\mathrm{H\bar{A}}$	—	157·0	24	109·1	1·2 $\mathrm{H\bar{A}}$	—	99·0
S10	Washed	0·5 $\mathrm{H\bar{G}}$ + 0·5 $\mathrm{H\bar{A}}$	—	113·2	—	107·2			
(see N 3)	pigeon	Control		6·0					
S11	Washed pigeon	0·5 $\mathrm{H\bar{G}}$ + 0·5 $\mathrm{H\bar{A}}$	—	188	46·2	182·0	0·5 $\mathrm{H\bar{G}}$ + 0·5 $\mathrm{H\bar{A}}$	—	144·6
S12	Pigeon +	1·0 $\mathrm{H\bar{A}}$	—	256·5	157·0				
	toluene	1·0 $\mathrm{H\bar{G}}$	—	109·0	0·0				
S13	Frog	0·5 $\mathrm{H\bar{G}}$ + 0·5 $\mathrm{H\bar{A}}$	—	181	45	152·5			
		1·0 $\mathrm{H\bar{G}}$	—	95·5	0	67·0			
		1·0 $\mathrm{H\bar{A}}$	—	151·0	50	122·5			
		Control		28·5					
S14	Washed	0·5 $\mathrm{H\bar{G}}$ + 0·5 $\mathrm{H\bar{A}}$	—	75·5	17·5	37·0			
	frog	1·0 $\mathrm{H\bar{G}}$	—	47·0	0	8·5			
		1·0 $\mathrm{H\bar{A}}$	—	35·4	0	0			
		Control		38·5					

Exps. S1, S2, S3, S4 and S6 were carried out by the earlier method for succinic acid only. In the other experiments the three acids together (succinic, malic and fumaric) were obtained as silver salts; the silver was estimated, and the result is expressed in the fifth column as succinic acid. The malic acid was also estimated separately in some cases. It is to be supposed that this malic acid has been formed, under the influence of fumarase, by hydration of fumaric acid, and that it is in equilibrium with the latter; as the equilibrium mixture consists of about 70 % malic and 30 % fumaric acid, about one and a half times the succinic acid equivalent of the malic acid figures must be subtracted from the total acid figures to obtain the true succinic acid values. The control value of Exp. S7 is used also for S8 and S9 in calculating the extra acid formed; the control value of S10 is used also for S11.

In all the experiments it appears very definitely that the addition of glutamic and aspartic acids together to the same muscle sample has a greater effect in increasing the succinic acid production than addition of an equivalent amount of either acid alone. The reason for this is not clear. Possibly two enzyme systems are concerned, one with glutamic acid and the other with aspartic acid breakdown; the fact that with washed frog's muscle aspartic acid is quite inactive, while glutamic acid gives rise to a marked succinic acid production and oxygen uptake, also suggests two separate systems. If each system is saturated when only 0·5 g. of the amino-acid is added to 100 g. muscle, then adding 1 g. would have no greater effect, and one would expect to obtain, by adding 0·5 g. of each to the same sample, the sum of the results obtained by adding 0·5 g. of each to two different samples. Actually, rather more than the sum is obtained; the simple hypothesis breaks down particularly, however, in the case of the washed frog's muscle, where, while aspartic acid alone is quite untouched, aspartic and glutamic acids together not only give a greater yield of total acids than does glutamic alone, but give a high percentage of malic acid. The figures obtained are not numerous enough for a detailed analysis of the results of adding the acids separately and together, or for the explicit formulation of any theory of multiple enzyme systems.

The highest yield of succinic acid obtained from the mixed amino-acids corresponds to about 37 % of the added amino-acids; with glutamic acid alone it corresponds to about 11 %, and with aspartic acid alone to about 15 %. When the malic acid is estimated, it is found to appear only after addition of aspartic acid or of aspartic and glutamic acids together; with glutamic acid alone no malic acid production could be found. Quastel and Woolf [1926], working with *B. coli*, showed that aspartic acid gives rise to ammonia and fumaric acid. In absence of growth inhibitors such as toluene, the fumaric acid is reduced to succinic; but the presence of toluene inhibits this reduction. It was now similarly found with muscle (see Exp. S12) that in presence of toluene, aspartic acid gave rise to a much higher percentage of malic acid—about 54 % instead of 20 %. The experiment was performed exactly as usual, except that to the buffer solutions 10 % by volume of toluene was added. Succinic acid only was obtained by adding glutamic acid in this experiment as in the others. It seems, then, that in the muscle succinic acid is formed directly from glutamic acid, while in the case of aspartic acid, fumaric and malic acids first appear, and are then reduced by reducing substances in the muscle to succinic acid.

In Exps. S10, S11 and S14, the muscle was washed (by shaking gently for 20 minutes in 10 volumes of ice-cold distilled water), and then strained through muslin. (The results given are per 100 g. of muscle before washing; the weight sometimes differed slightly after the process.) In the case of the pigeon, washing did not very greatly inhibit the production of succinic, malic and fumaric acids from glutamic and aspartic, although the muscle alone showed practically no production; with the frog, production from the muscle alone seemed to be unaffected, and some increased production took place from added glutamic, and from the two amino-acids together, but not from aspartic acid.

Table II.

No. of exp.	Material	Acid added g. per 100 g. muscle	Total acids as mg. succinic acid per 100 g.	Malic acid mg. per 100 g.
O 1	Pigeon	$2\ H\overline{A}+2\ H\overline{G}$	49·6	36
		Control	10·8	2
O 2	Pigeon	$2\ H\overline{A}+2\ H\overline{G}$	27·2	12
		Control	9·28	—
O 3	Pigeon	$5\ H\overline{\overline{S}}$	3·070	2·700
		$5\ H\overline{\overline{F}}$	3·070	2·800
		$3\ H\overline{G}+3\ H\overline{A}$	350	370
O 4	Pigeon (washed)	$5\ H\overline{\overline{S}}$	3840	3400
		$5\ H\overline{\overline{F}}$	3840	3600
		5 acetic acid	30	

A few experiments were also carried out on the progress of events in oxygen (Table II). Here small but definite amounts of the total acids were obtained; in the case of the controls these seemed to consist almost entirely of succinic, but in the samples to which glutamic and aspartic acids had been added, they consisted partly or wholly of malic and fumaric acids. In Exp. O 1 the muscle was suspended in 4 volumes of 1 % NaCl at 35° for 6 hours; oxygen was bubbled through the bottles, which were shaken at intervals. In Exp. O 2 the conditions were the same except that the bottles were filled with oxygen and shaken on a shaking machine; at the end of the experiment the muscle appeared brown and coagulated, perhaps owing to too violent shaking. In Exp. O 3 the muscle was suspended in 5 volumes of 1·4 % phosphate, the bottles were filled with oxygen and shaken very gently in a Barcroft-bath. In order to compare what happened in similar circumstances with succinic and fumaric acids, these acids also were added to certain samples. With the unwashed muscle it is seen that, in both cases, about 60 % of the original amount added is recovered. As the method gives results 85 % of the theoretical, this means that 70 % remains after the oxidation time and about 30 % has been oxidised away. This 70 % consists entirely of malic and fumaric acids. With the washed muscle, 77 % (corrected, 90 %) is recovered, and this also consists entirely of malic and fumaric acids.

The free acid was in several cases prepared from the silver salts, as described in an earlier paper [Moyle, 1924]. The acid obtained, after addition of glutamic and aspartic acids, gave a sharp melting-point at 180–181°, when the method including permanganate oxidation was used. When the method giving the total silver salts was used, the acid prepared from these showed partial melting at 180°, which was not complete until 200°. Its behaviour resembled that of a mixture of succinic and fumaric acids. A sample of these "total acids" was tested for succinic acid in the following way. It was treated with five times its weight of *p*-toluidine, and heated for half-an-hour at 220°. The resulting mixture was treated with 50 % alcohol, and the crystalline precipitate of succinotoluidide was filtered off and washed. It was recrystallised from boiling alcohol, washed and dried at 100°; M.P. 258°.

THE FATE OF THE AMINO-GROUP.

The attempts to find changes in the soluble nitrogen fractions during the production of succinic, malic and fumaric acids from glutamic and aspartic acids are summarised in Table III.

Table III.

No. of exp.	Material	Acid added g. per 100 g.	Ammonia-N mg. per 100 g.	Urea-N mg. per 100 g.	Uric acid mg. per 100 g.	Amide-N mg. per 100 g.	
N 1 (see S 7)	Pigeon (anaerobic)	1·0 HĞ + 0·88 HA	14·5	0			
		Control	13·0	0			
N 2	Pigeon (anaerobic)	1·0 HĞ + 1·0 HĀ	16·5	0	2·75		
		Control	16·5	0	2·12		
N 3 (see S 10)	Washed pigeon (anaerobic)	1·0 HĞ + 1·0 HĀ	0·2	0	—	12	
		Control	0·2	0·2	—	11	
N 4	Pigeon (anaerobic)	1·2 HĞ + 15·2 mg. ammonia-N	26·1	—	—	—	85 % added NH_3 recovered; none formed from HĞ
		Control	13·2				
N 5	Pigeon (anaerobic)	0·6 HĞ + 0·5 HĀ + 13·7 mg. ammonia-N	21·8	—	—	—	75 % added NH_3 recovered; none formed from HĞ and HĀ
		Control	11·5				
N 6	Pigeon (oxygenated)	0·66 HĞ + 0·66 HĀ	20·6	—	—	—	6 mg. per 100 g. NH_3-N formed
		Control	14·7				

The ammonia and urea were estimated in the first experiments (N 1, N 2 and N 3) by Folin and Wu's [1919] method; in the later experiments (N 4, N 5 and N 6) the ammonia was estimated by a modification of Stanford's method [1923]. The entire sample (about 2·5 g.) in 2 vols. of phosphate solution was washed into the distilling flask; saturated borate solution was used instead of soda. The vacuum distillation was carried out at 45° and 15 mm. pressure; for 5 minutes the apparatus was shut off from the pump, then for 5 minutes connected with it. For 10 minutes a very slow stream of ammonia-free air was drawn through. Under these conditions, with the original volume of liquid in the flask about 30 cc., 80 % of the ammonia present could be estimated. This yield is low but quite constant, and the method has the advantage of working at a comparatively low temperature.

When an extract of the muscle was used, in order to make sure of extracting the ammonium salts without driving off any ammonia, the samples were either (1) treated with alcohol containing enough HCl to bring the final p_H to about 6·0, the alcohol being afterwards removed; or (2) treated with dilute HCl to bring to p_H 6·0–6·5, and coagulated by plunging the vessels into a boiling water-bath. This technique was only used if the samples were small, and therefore could be almost instantaneously coagulated by heat.

Uric acid was estimated by Benedict and Franke's method [1922], using the slightly acid (p_H 6·0) aqueous extract. This was neutralised and evaporated and traces of protein were filtered off.

For the amide estimations the samples after incubation were washed into round-bottomed flasks and rapidly coagulated in a boiling water-bath. HCl was added to make the concentration 5 % by volume, and the contents of the flasks were hydrolysed for 2 hours, under reflux condensers. At the end of this time they were partly neutralised with Na_2CO_3. The ammonia present was estimated by Folin and Wu's method, and the amide-nitrogen found by subtracting the ammonia present before hydrolysis.

Table IV.

No. of exp.	Material	Acids added g. per 100 g.	Van Slyke-N mg. per 100 g.	Average values mg. per 100 g. for amino-N of muscle alone
N 7	Pigeon	0·44 $H\bar{G}$ + 0·44 $H\bar{A}$ (amino-N 88·8 mg.)	143	52·7
	(anaerobic)	Control	145	
	(oxygenated)	0·44 $H\bar{G}$ + 0·44 $H\bar{A}$ (amino-N 88·8 mg.)	139	
		Control	138	
N 8 (see S 11)	Washed pigeon	0·54 $H\bar{G}$ + 0·54 $H\bar{A}$ (amino-N 108·5 mg.)	133·6	15
	(anaerobic)	Control	116·4	
	(aerobic)	0·54 $H\bar{G}$ + 0·54 $H\bar{A}$ (amino-N 108·5 mg.)	117	
		Control	128	
N 9	Washed pigeon	0·48 $H\bar{G}$ + 0·48 $H\bar{A}$ (amino-N 96 mg.)	114·4	18
	(oxygenated)	Control	113·0	
N 10 (see B 2)	Washed pigeon	0·5 $H\bar{G}$ + 0·5 $H\bar{A}$ (amino-N 100·5 mg.)	94	13·5
	(aerobic)	Control	122	
		$H\bar{A}$ 1 g. (amino-N 105 mg.)	129·8	
		Control	118·0	
N 11 (see A 3 and B 19)	Pigeon	1 g. $H\bar{A}$ (amino-N 105 mg.)	175·8	69·3
	(aerobic)	Control	172·8	
N 12	Pigeon	1 g. $H\bar{A}$ (amino-N 105 mg.)	179·8	67·9
	(oxygenated)	Control	166·0	

For the estimations of free amino-nitrogen a modification of Parnas and Wagner's procedure [1914] was used. The samples were first coagulated in a boiling water-bath; they were cooled in ice, and HCl was added to make the concentration 0·25 % (by volume). The flasks stood at 0° overnight; next day the muscle was strained off, ground with more 0·25 % acid, and strained again. The grinding and straining was repeated once more; the filtrate was filtered through paper, neutralised and evaporated. It was centrifuged, and the clear centrifugate was made up to a known volume, of which parts were used for estimations in the Van Slyke apparatus. In some cases, any soluble protein was removed by adding trichloroacetic acid to a concentration of 4 % before centrifuging. This treatment did not seem to affect the results.

In all the experiments in which nitrogenous fractions were to be estimated, the p_H of the buffer solution in which the muscle samples were suspended was 7·0–7·4; 1·4 % $Na_2HPO_4, 2H_2O$ was taken and brought to the required p_H by adding phosphoric acid. For the anaerobic and some of the aerobic experiments, 2 volumes of buffer solution were used; for the later aerobic experiments (N 9, N 12), 5 volumes were used. In all the aerobic experiments the vessels used contained a large volume of air or oxygen, which was renewed at intervals, and the vessels were shaken gently. The experiments lasted from 3½ to 5 hours at 37°.

Exp. N 1 was performed on the same material as S 7; N 3 on the same material as S 10, and N 8 on the same material as S 11. For Exp. N 10 the material from the Barcroft cups of B 2 was used, and for Exp. N 11 the material from B 19.

No increase in ammonia could be shown under anaerobic conditions, in washed or unwashed muscle. The value obtained, about 14 mg. per 100 g., agrees satisfactorily with the maximum found for pigeon muscle by Parnas and Mozolowski [1927]. Control experiments showed that small amounts of ammonia, such as those equivalent to the amounts of extra succinic acid formed in the samples, added (as $(NH_4)_2SO_4$) to muscle could be quantitatively recovered. It was thought possible that, in presence of excess of glutamic and aspartic acids, there might be combination between ammonia and these acids, whether as amide or in some other form. The ammonium salt was therefore added in presence of the glutamic and aspartic acid, but in these circumstances also could be quantitatively recovered.

In oxygen a small liberation of ammonia took place; it amounted only to about 5 % of that contained in the added amino-acids.

No increase in urea, uric acid or amide-nitrogen was found.

In the Van Slyke experiments, the same amounts of glutamic and aspartic acids were added to the control samples immediately before the flasks were washed out into the coagulation vessel. In some cases, the amino-nitrogen was estimated on the muscle which had been in the Barcroft apparatus, and of which the actual oxygen uptake was known. No significant difference could be found between the results from the samples incubated with the amino-acids (either with or without oxygen for several hours), and those to which the amino-acids were added at the last moment. Control experiments showed that these two amino-acids are quantitatively extracted and estimated by this method, and the values obtained (by subtraction) for the amino-nitrogen of the muscle alone closely resemble those found by Van Slyke [1913–14] for dog muscle and by Parnas and Wagner [1914] and Meyerhof [1924] for frog muscle.

OXYGEN UPTAKE ON ADDING GLUTAMIC AND ASPARTIC ACIDS.

The measurements of oxygen uptake were made with Barcroft differential manometers and the results are given in Table V. The oxygen uptake of the muscle alone was measured by placing in one cup 1·5 cc. buffer solution and 1·5 cc. water; in the other, 1·5 cc. buffer solution, 0·5 g. of muscle and 1 cc. of water. To find the increased uptake due to addition of the amino-acids, 1·5 cc. of buffer solution and 0·5 g. of muscle were placed on each side, with 1 cc. of water on one side and 1 cc. of the amino-acid solution (neutralised with NaOH) on the other. The muscle was added to the solutions at the last moment before the cups were attached; the apparatus was shaken in the bath for 15 minutes with the taps open. The taps were then closed, and readings were made at intervals. In the earlier experiments, 10 drops of 40 % potassium hydroxide were placed in the central cup for absorbing carbon dioxide; in later experiments, on the advice of Dr M. Dixon, a roll of filter paper soaked in 5 % KOH was placed instead in the cup, and projected from it. The bath was kept at 37° for the pigeon muscle, and at 20° for frog muscle. The oxygen uptake of the pigeon muscle alone fell off rapidly after about 3½ hours; by the time an almost steady state was reached the oxygen taken up usually amounted to about 1800 mm.^{3} for 0·5 g. muscle, although with some samples it was as high as 2400, and with others as low as 500, for no obvious reason. The increased uptake found in the presence of glutamic and aspartic acids usually varied with the respiratory activity of the muscle alone, being greater if the control respiration was high and lower if the control respiration was feeble. The calculated oxygen required for oxidation of 5 mg. glutamic acid to ammonia, carbon dioxide and water is 3330 mm.^{3}; for 5 mg. of aspartic acid it is 2220 mm.^{3}: for half quantities of each, 2775 mm.^{3} When 5 mg. of the amino-acid was added, the increased oxygen uptake during the time of the experiment usually amounted to about 1500 mm.^{3}, two-thirds to one-half of the calculated maximum. The extra uptake usually began very slowly, and after about 1 hour increased rapidly, to fall off again in another 2 hours. When the experiment was stopped, the uptake usually continued, but at a very slow rate. When 10 mg. of acid was added, about the same oxygen uptake occurred in the time, but from the shape of the curve was obviously quite incomplete. When 1 mg. was added, oxygen uptake practically came to a standstill by the end of the experiment. Here there was usually enough oxygen absorbed to account for complete oxidation, and sometimes even larger quantities were absorbed (see Figs. 1 and 2). These last experiments may not be very reliable; the oxygen uptake of the muscle alone is so much greater than the increase caused by addition, that small

Table V.

No. of exp.	Tissue	Acids added mg.	Total amount of extra O_2 uptake mm.^{3}	Control O_2 uptake mm.^{3}	Duration hrs.
B 1	Washed pigeon	2·5 $H\bar{G}$; 2·5 $H\bar{A}$	68·2		4½
	(10 vols. 20 min.)	5 $H\bar{G}$	13·1		
		5 $H\bar{A}$	9·5		
		Control	—	104·2	
B 2	Washed pigeon	2·5 $H\bar{G}$; 2·5 $H\bar{A}$	272·5		4
(see N 10)	(10 vols. 1½ min.)	5 $H\bar{G}$	114·4		
		5 $H\bar{A}$	286·0		
		Control	—	284·4	
B 3	Pigeon	2·5 $H\bar{G}$; 2·5 $H\bar{A}$	1478		3¾
		5 $H\bar{G}$	1418		
		Control	—	1905	
B 4	Pigeon	5 $H\bar{A}$	1257		4¾
		1 $H\bar{A}$	637		
		Control	—	861	
B 5	Pigeon	5 $H\bar{G}$	2072		4¾
		Control	—	2376	
B 6	Pigeon	5 $H\bar{A}$	518		
		1 $H\bar{A}$	336		
		Control	—	561	
B 7	Pigeon	10 $H\bar{G}$	1743		5¼
		5 $H\bar{G}$	1673		
		1 $H\bar{G}$	709		
		Control	—	2013	
B 8	Pigeon	1 $H\bar{A}$	747		3¾
		1 $H\bar{G}$	452		
		Control	—	1570	
B 9	Washed pigeon	5 $H\bar{A}$	49·1		
	(10 vols. 20 min.)	5 $H\bar{G}$	35·2		
		Control	—	194	
B 10	Pigeon	0·5 $H\bar{G}$ + 0·5 $H\bar{A}$	636·0		5¾
		Control	—	1848	
B 11	Frog washed	5 $H\bar{A}$	0		5
	(10 vols. 20 min.)	5 $H\bar{G}$	194		
		2·5 $H\bar{G}$ + 2·5 $H\bar{A}$	127·5		
		Control	—	30·6	
B 12	Frog washed	3 $H\bar{G}$	246		7
	(10 vols. 20 min.)	2 $H\bar{G}$	182·8		
		1 $H\bar{G}$	147·5		
		Control	—	57	
B 13	Pigeon washed	5 $H\bar{G}$	285·5		5½
	(10 vols. 20 min.)	5 $H\bar{A}$	87·0		
		2·5 $H\bar{G}$ + 2·5 $H\bar{A}$	123·5		
		Control	—	414	
B 14	Pigeon washed	5 $H\bar{G}$	31·7		
	(10 vols. 20 min.)	5 $H\bar{A}$	0		
		2·5 $H\bar{G}$ + 2·5 $H\bar{A}$	0		
		Control	—	146·0	
B 15	Frog washed*	5 $H\bar{G}$	32·9		6
		5 $H\bar{A}$	0		
		Control	—	0	
B 16	Pigeon washed*	+5 $H\bar{G}$	24·7		
		+5 $H\bar{A}$	0		
		Control	—	59·2	
B 17	Pigeon	5 $H\bar{G}$	1917		
		5 $H\bar{A}$	1626		
		Control	—	2495	
B 18	Frog	1 $H\bar{G}$	245·2		19¾
		0·5 $H\bar{G}$	110·1		
		Control	—	180·5	
B 19	Pigeon	5 $H\bar{A}$	1399	—	4¾

* Six times with 10 volumes of phosphate solution (48 g. $Na_2HPO_4, 2H_2O$ + 54 g. KH_2PO_4 per litre) [see Hahn and Haarmann, 1928].

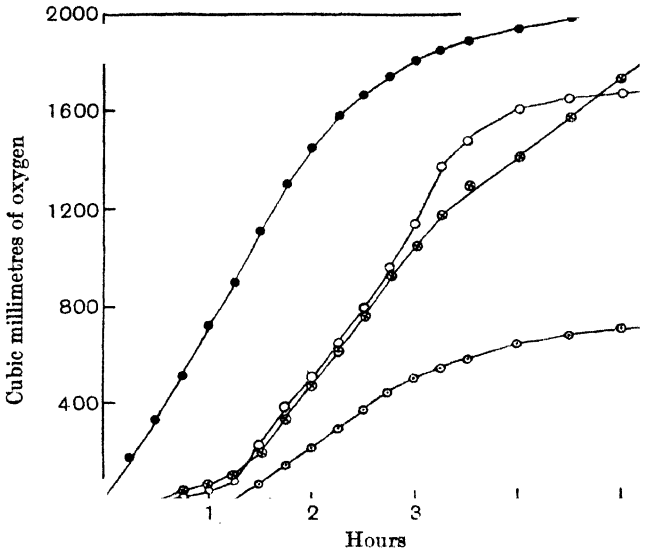

Fig. 1.

——●——●—— Uptake of control.
——○——○—— Extra uptake with 5 mg. glutamic acid.
——⊗——⊗—— Extra uptake with 10 mg. glutamic acid.
——⊙——⊙—— Extra uptake with 1 mg. glutamic acid.

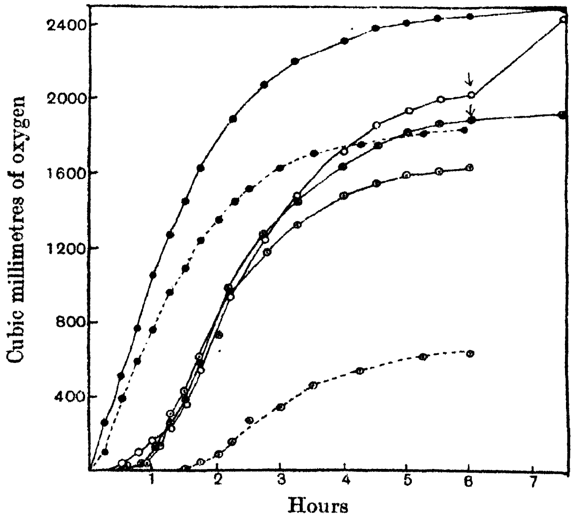

Fig. 2.

——●——●—— Uptake of control.
——○——○—— Extra uptake with 5 mg. succinic acid; 5 mg. more added at ↓.
——⊗——⊗—— Extra uptake with 5 mg. glutamic acid; 5 mg. more added at ↓.
——⊙——⊙—— Extra uptake with 5 mg. aspartic acid. Exp. B 10.
– – –●– – –●– – – Uptake of control.
– – –⊙– – –⊙– – – Extra uptake with 0·5 mg. aspartic acid + 0·5 mg. glutamic acid.

variations in the amount of muscle tissue in the different cups, or small variations in the degree of chopping, might have large effects.

Attempts to reduce the control respiration without too much diminishing the effect of adding the amino-acids were unsuccessful in the case of the pigeon; the oxidation of the added amino-acid was usually reduced to a greater extent than the control respiration, and in some experiments was almost absent. Addition of the two amino-acids together seemed to have no greater effect than addition of either alone with the washed or unwashed muscle.

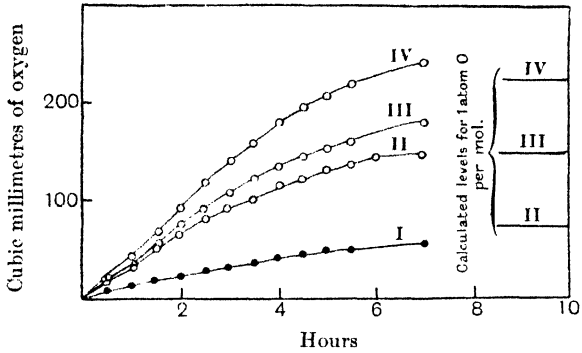

Fig. 3.

I. Uptake of control.
II. Extra uptake with 1 mg. glutamic acid added.
III. Extra uptake with 2 mg. glutamic acid added.
IV. Extra uptake with 3 mg. glutamic acid added.

Frog muscle (as was suggested to me by Dr D. C. Harrison) proved much more satisfactory material for a study of the oxygen uptake. By washing once with 10 volumes of distilled water the control respiration falls to about 40 mm.^{3} for 0·5 g. at 20° for 7 hours, while the increased uptake due to the added amino-acid is five or six times this amount. With washed frog muscle, aspartic acid had no effect. The rate of oxygen uptake varied according to the concentration of added glutamic acid, but the relative absorption was greatest with the smallest amounts (see Fig. 3). The highest was 3 atoms of oxygen per molecule when 1 mg. of glutamic acid was added.

OXYGEN UPTAKE WITH OTHER AMINO-ACIDS AND AMMONIUM SALTS.

In Table VI are given the results of adding some other amino-acids and also ammonium chloride to unwashed pigeon muscle. With 1·75 mg. ammonium chloride, nearly 30 % inhibition of the muscle's own respiration was found; with a tenth of this amount only slight inhibition took place. In these two experiments an equivalent amount of sodium chloride was placed in the opposite Barcroft cup. Glycine, alanine, valine, leucine and tyrosine all showed inhibitory action, sometimes to a very marked degree (see Fig. 4). Glutamic and aspartic acids thus seem to occupy a very special position with regard to muscle.

Table VI.

No.	Material	Substances added	Oxygen uptake	Inhibition %	Duration hrs.
B20	Pigeon	NH_4Cl (1·75 mg.)	1760	27	5
		Control	2274		
B21	Pigeon	NH_4Cl (0·18 mg.)	817	5	4
		Control	861		
B22 (see B8)	Pigeon	1 mg. glycine	1381	12	4
		Control	1570		
B23	Pigeon	5 mg. alanine	1433	33	3½
		1 mg. alanine	1743	18	
		Control	2125		
B24	Pigeon	5 mg. leucine	890	35	5½
		5 mg. valine	448	67	
		Control	1378		
B25	Pigeon	5 mg. leucine	983	9	4¼
		5 mg. valine	886	18	
		Sat. sol. tyrosine	799	26	
		Control	1080		

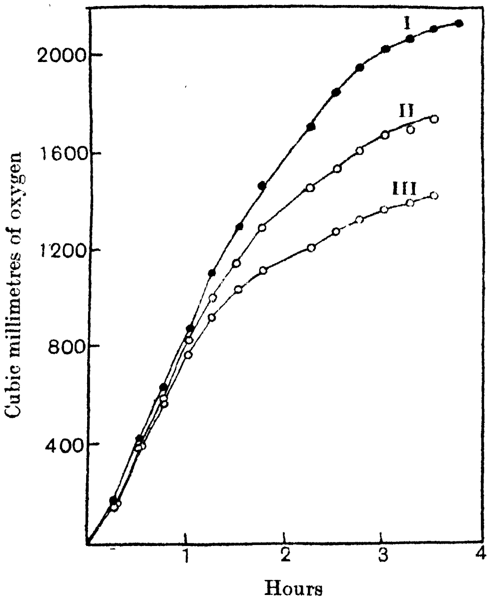

Fig. 4.

I. Uptake of control.
II. Uptake after addition of 1 mg. alanine.
III. Uptake after addition of 5 mg. alanine.

DISAPPEARANCE OF ADDED ASPARTIC ACID.

These experiments were carried out in order to ascertain whether, under conditions such that a marked increase in oxygen uptake would occur, the added aspartic acid showed any corresponding decrease in amount.

I am indebted to Mr B. Woolf for the suggestion that the aspartic acid might be estimated by conversion into malic acid, as a method was available for estimation of the latter in muscle. A large number of preliminary experiments showed that with $NaNO_2$ and acetic acid at room temperature or higher deamination was complete, but a large part of the equivalent malic acid had disappeared, presumably by oxidation. The best results were finally obtained in the following way. 10 cc. of the neutral solution containing aspartic acid cooled to 0° were treated with 1 cc. 30 % $NaNO_2$ and 0·25 cc. 50 % H_2SO_4. The mixture was kept at 0° over night, being shaken well at frequent intervals during the first hour. Deamination was then found to be practically complete, and 80 % of the calculated malic acid was found.

The experiments were carried out in the following way. Five g. samples of minced muscle were suspended in 25 cc. of 1·4 % phosphate in bottles of 250 cc. capacity. One bottle (A) contained 50 mg. of aspartic acid. All were shaken very gently, in a horizontal position, at 37° for 4½ hours. The bottles were opened for re-aeration every half hour. At the end of the time one bottle (C +) received 10 cc. solution containing 50 mg. aspartic acid, and 10 cc. water was added to the third (C). Each sample was immediately washed into a boiling-tube, and coagulated in a boiling water-bath. The slightly acid extract was prepared, neutralised and concentrated as in the Van Slyke experiments. To the clear solution, measuring 10 cc., 1 cc. $NaNO_2$ and 0·25 cc. 50 % H_2SO_4 were added at 0°, as described. The next day the excess of nitrous acid was removed by adding 0·3 g. of urea, and shaking till effervescence had subsided. The solutions were neutralised and made up to 25 cc. They were placed in covered flasks on a boiling water-bath; 5 g. uranium acetate was added, and the flasks were heated for 20 minutes. The contents were cooled in the dark and filtered. The rotation was taken, using the green line of the mercury lamp. Control experiments with malic acid solutions containing phosphate and treated with uranium acetate in this way gave $[\alpha]^{20°}_{5461} = -430°$.

Experiment A 1.

	Readings	Change in rotation	% recovered
Zero	1·4°		
C	1·5		
C +	0·37	1·13	64
A	0·95	0·55	32

These figures show that only about half the aspartic acid is present after the incubation.

An attempt was made to get conditions on the large scale more resembling those in the Barcroft cups. Five g. samples of muscle in 25 cc. of solution were placed in litre Roux bottles, the flat surface of which was about 220 sq. cm., or ten times the area of the bottom of a Barcroft cup. A stout wire passed through the rubber stopper of each Roux bottle, holding in the centre of the bottle a porcelain boat. The boat contained filter paper soaked with 5 % KOH. In order to promote oxidation, the bottles were filled with oxygen before the experiment began, then they were all placed horizontally in a bath at 37°, attached to an overhead bar, which moved gently backwards and forwards. The final solutions were made up to 15 cc.

Experiment A 2.

	Reading	Change in rotation	% recovered
Zero	3·24°		
C	3·30		
C1 +	1·48	1·82°	63
C2 +	1·60	1·7	61
A	2·10	1·2	42

Here about two-thirds of the aspartic acid remain.

In a third experiment the procedure was exactly as just described, but the muscle was chopped with fine scissors as for the Barcroft experiments, instead of being minced. A sample was used for oxygen uptake estimation (see B19), and the contents of the Barcroft cups were used afterwards for Van Slyke estimations (see N11). The solutions were made up to 15·5 cc., and sodium light was used. $[\alpha]_D^{20°}$ was found under these conditions to be − 350°. The solution is acid, and the value agrees with that (− 345°) found by Itzig [1901] for the compound $UrO_3C_4H_6O_5$. Addition of alkali, as observed by Walden [1897], increases the rotation to a maximum; almost the same maximum (− 512°) was found by Walden for the compound $UrO_3C_4H_5O_5Na$.

Experiment A 3.

	Reading	Change in rotation	% recovered
Zero	3·4°		
C1+	5·0	1·6°	71
C2+	4·9	1·5	66
A1	4·4	1·0	44
A2	4·3	0·9	40

Here about three-sevenths of the aspartic acid has disappeared; the oxygen uptake observed in the time was 1399 mm.^{3}, or almost two-thirds of the amount needed for complete oxidation; the amino-nitrogen was unchanged.

Discussion.

The results described in this paper make it seem very probable that, if free glutamic and aspartic acids are present in the muscle or are brought to it in the blood stream, then production of succinic, fumaric and malic acids must follow, and also an increase in oxygen uptake, probably due to oxidation of these three acids (although there is no evidence that all the glutamic or aspartic acid oxidised passes through the stage of a 4-carbon non-amino-acid).

A comparison of the oxygen uptake curve obtained when glutamic or aspartic acid is added to unwashed pigeon muscle with that obtained when succinic acid is added, shows that the two curves are very similar (see Fig. 2). The chief differences are, firstly, that in the latter case the uptake persists rather longer, and practically the calculated total is absorbed; and secondly that, when the absorption has come to a standstill, it is possible, by adding more succinic acid, to call forth another marked oxygen absorption, while addition of glutamic acid has no effect. This suggests that the mechanism responsible for the conversion of the amino-acid into succinic or fumaric acid gradually fails, and that under the conditions of the experiment it has failed before complete conversion has had time to take place. If we now consider the aerobic experiments on the large scale, we find, in the first place, that less aspartic acid disappeared (only one-half to one-third) than one would expect from the Barcroft experiments (one-half to two-thirds). Secondly, in Exp. O3, with added succinic acid, a large quantity of fumaric and malic acids appeared at the end of the experiment, while, from the same amount of glutamic and aspartic acids, only about one-ninth of the amount of fumaric and malic acids appeared. These facts might again be explained by the comparatively early failure of the enzyme system producing succinic and fumaric from glutamic and aspartic acids; conditions on the large scale are probably less favourable than in the Barcroft cups. The succinic and fumaric acids formed during the early period of the experiment would gradually be oxidised further. One must not, however, lose sight of the possibility that, even under aerobic conditions, only about 25 % of the amino-acids passes through succinic acid, the rest following some other path of breakdown.

As regards the possible occurrence of the amino-acids in question in the free state in muscle, Osborne and Heyl [1908] for chicken muscle and Osborne and Jones [1909] for ox muscle have shown that glutamic acid constitutes about 16 % of the protein and aspartic acid about 4 %. Micko [1908] found free glutamic acid, alanine and taurine in meat extract, but figures for the free amino-acids of fresh muscle do not seem to be available. There is 0·07 % of amino-nitrogen present in the muscle, and this is probably largely contained in free amino-acids; for the nitrogen of creatine and methylguanidine is not estimated by Van Slyke's method [Van Slyke, 1911], and there are only traces of carnosine present in pigeon muscle [Clifford, 1921]. The fact that washed frog muscle gives the same sort of yield of succinic acid as unwashed muscle, while formation of succinic acid does not take place in washed pigeon muscle (although it retains the power to act on added glutamic and aspartic acids), may be due to the greater acidity developed in a short time by the pigeon muscle. This might enable the content of amino-acids to be washed out of the pigeon muscle, while in the frog muscle they might not be extracted. The fact that, in the silver salts obtained from the muscle alone, malic acid was either absent or present only in traces, may point to the origin of the succinic acid from glutamic acid alone, unmixed with aspartic.

It was shown by Thunberg [1920–21] that, if glutamic acid is added to washed frog muscle in presence of methylene blue, the methylene blue is reduced. Alanine alone of the other amino-acids used gave a similar but much slower decoloration. Thunberg postulated oxidative deamination. A little later, Abderhalden and Wertheimer [1921] found that addition of glutamic acid increases the oxygen uptake of red blood cells, muscle and kidney. The other amino-acids tried had no effect; aspartic acid was tested on the red cells, though not on muscle. Ahlgren [1924] showed that, although washed frog's muscle does not activate aspartic acid, yet in the presence of unwashed muscle the reduction of methylene blue is accelerated by addition of aspartic acid. The appearance of an equilibrium point at partial reduction of methylene blue was reminiscent of the behaviour of malic or fumaric acid, and he was of opinion that hydrolytic deamination with formation of malic acid took place. Neither Thunberg nor Ahlgren investigated the question of ammonia formation. As we have seen, the results of the present work show that no simple deamination process occurs, either under aerobic or anaerobic conditions. This agrees with the experience of several workers who have found that sterile tissues or tissues with toluene have no power of producing ammonia from amino-acids. For example, Levene and Meyer [1913, 1, 2] were unable to find ammonia formation when aspartic acid, leucine, glycine or alanine was added to sterile leucocytes or kidney tissue. Luck [1924], using suspensions of various mixed tissues with toluene and hydrolysed gelatin or caseinogen as substrate, found no evidence for the formation of ammonia at the expense of free amino-nitrogen. That ammonia formation may take place in some cases on addition of amino-acids to sterile tissue has, on the other hand, been shown by Patey and Holmes [1929] for glycine in the presence of washed kidney. That in this case also there is not a straightforward oxidative deamination is shown by the fact that, although oxygen is necessary for the reaction, an oxygen absorption corresponding to the ammonia production could not be demonstrated.

As a hypothesis which would not contradict the facts described in this paper we might consider the possibility of the linkage of the NH_2-group of the glutamic acid with some reactive carbohydrate residue in the muscle; when oxidation and splitting off of the nitrogen with production of succinic acid occurs, the nitrogen is left in combination with this carbon chain, a new α-amino-acid thus being formed.

It is well known that the oxidation of amino-acids *in vitro* may take different courses according to the presence or absence of carbohydrate substances. Fearon and Montgomery [1924] have pointed this out for the oxidation of glycine with dilute permanganate; more cyanic acid is formed and less ammonia when formaldehyde or glucose is present, and Fearon suggests that this result is due to combination between the NH_2-group of the glycine and the aldehyde radical.

As regards the ammonia production by tissues, several workers have found carbohydrate or similar compounds to have an inhibitory effect upon this. Warburg, Posener and Negelein [1924], using rat carcinoma and retina, showed that both the anaerobic and the aerobic formation of ammonia were reduced to about one-tenth of their value by addition of glucose. Meyerhof, Lohmann and Meier [1925] found that the diaphragm muscle of starved rats produces ammonia in aerobic conditions, and this ammonia production is greatly lessened by adding glucose, lactic acid or pyruvic acid. Watchorn and Holmes [1927] found less ammonia and urea formed during growth of tissue cultures when the medium contained glucose; and Patey and Holmes [1929], using washed kidney tissue, found that the aerobic (though not the anaerobic) ammonia production was much less in presence of glucose. The ammonia production from added glycine was not affected. If, in the case of glutamic and aspartic acids, in muscle, the presence of carbohydrate or its breakdown products prevents the appearance of free ammonia, then this prevention goes on anaerobically as well as in oxygen. This behaviour of certain carbon compounds in lessening ammonia formation is usually regarded as indicating "protein-sparing action"; in the present case, where it is postulated that one amino-acid is formed from another, part of the latter being oxidised, it would more correctly be described as "nitrogen-sparing."

It has been suggested by Thunberg [1920–21] that succinic acid might arise by removal of hydrogen atoms from two acetic acid molecules, and condensation of the residues. If this were the case, succinic acid might play an important part in fat metabolism, and also, as Wieland [1922] has pointed out, in carbohydrate metabolism. Lactic acid may give rise to pyruvic acid, and the latter by decarboxylation and oxidation to acetic acid. Very recently it has been shown by Butkewitsch and Fedoroff [1929] that, in presence of the mould *Mucor stolonifer*, acetic acid may be converted, to the extent of 15–30 %, into succinic and fumaric acids.

It has never, however, been possible to show that acetic acid can give rise to succinic acid in muscle. With unwashed muscle, Clutterbuck [1927] failed to find any increase in malic acid following upon incubation with acetate in oxygen; and I have similarly failed to find any increase with washed muscle. This does not prove that the condensation does not take place; in unwashed muscle a sufficiency of acetic acid may always be present, so that addition of more has no effect, while with washed muscle some part of the enzyme system responsible may have been removed.

Clutterbuck and Raper [1925, 1] have shown that γ-oxidation of fatty acids may take place with hydrogen peroxide *in vitro*, and they suggest that succinic acid may be formed in this way in the body; their experiments, however, on injecting β-phenylhexoic acid, gave negative results, no phenylsuccinic acid appearing in the urine [1925, 2].

In conclusion, it may be said that, although formation of succinic acid from glutamic and subsequent oxidation of the succinic acid is probably a part of muscle metabolism, yet it is not suggested that this is the only origin of the succinic acid in the body, or that the only function of the "succinoxydone" is to oxidise an intermediate stage in the breakdown of these amino-acids. Under anaerobic conditions, succinic acid formation seems to be a self-hindered reaction; under aerobic conditions, the acid is oxidised away and its formation cannot be measured; it may therefore play some as yet undetected part in carbohydrate and fat metabolism *in vivo*.

SUMMARY.

1. It has been shown that glutamic and aspartic acids, when added to minced muscle under anaerobic conditions, give rise to an increased amount of succinic, fumaric, and malic acids. The maximum amount of extra acid obtained was about 37 % of the equivalent of the amino-acids when both were added together; about 15 % when aspartic acid alone was added; and about 11 % when glutamic acid alone was added. From the mixed amino-acids or from aspartic acid alone, malic and fumaric as well as succinic acid were formed; from glutamic acid only succinic.

2. Ammonia was not produced during this formation of succinic, fumaric, and malic acids; nor could any increase of urea, uric acid, or amide-nitrogen be found. The amino-nitrogen (estimated by Van Slyke's method) at the end of the experiments was equal to that of the controls plus that of the added amino-acid.

3. An increased oxygen uptake was found when glutamic or aspartic acid was added to minced muscle. This uptake usually corresponded to one-half or two-thirds of the calculated amount for complete oxidation. The other amino-acids tried (glycine, alanine, valine, leucine and tyrosine) had an inhibitory effect on the muscle's own respiration.

4. Under aerobic conditions traces of malic and fumaric acids were found after addition of glutamic and aspartic acids. No change in the soluble nitrogen fractions was found.

5. A method for estimation of aspartic acid was worked out, and by its aid it was found that after incubation with muscle in air or oxygen one-half to two-thirds of the aspartic acid had disappeared.

6. It is suggested that possibly a combination of the amino-group with some reactive carbohydrate residue takes place; then when splitting and oxidation occur the amino-group is retained in the form of a new amino-acid.

I am very grateful to Miss M. Stephenson and Dr J. Needham for advice in practical details and to Sir F. G. Hopkins for his continual interest and encouragement.

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Dinner given by the Biochemical Society

ON FEBRUARY 3RD, 1930

in honour of the Nobel Laureates in Chemistry and Medicine 1929

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OBITUARY NOTICE.

SAMUEL BARNETT SCHRYVER (1869-1929).

THE death of Samuel Barnett Schryver, which occurred on August 21st, 1929, after an illness of several months' duration, has deprived Biochemistry of an active worker, possessed of a mind fertile in original ideas, and still at the height of intellectual activity. Schryver was an original member of the Biochemical Society, in the affairs of which he always took a great interest, and he frequently presided over the meetings held in his laboratory at South Kensington. Schryver was born in London in 1869 and was educated at University College School, University College and the University of Leipzig where he graduated Ph.D. After his return to England he was appointed in 1893 Demonstrator in Chemistry under Campbell Brown at University College, Liverpool, where he remained until 1897. Early in 1898 he joined the staff of the Wellcome Research Laboratory and retained this position until March 1901, when he was appointed Lecturer in Physiological Chemistry at University College, London. Up to this time he had been engaged in research on a variety of subjects in Organic Chemistry, but here for the first time he came into direct contact with biochemical problems and henceforward until his death he was continuously engaged in biochemical and physiological research. He left University College in 1907, on his appointment as Physiological Chemist to the Research Institute of the Cancer Hospital and in 1913 joined the Staff of the Imperial College of Science at South Kensington as Assistant Professor, becoming full Professor of Biochemistry in 1920. He was elected to the Fellowship of the Royal Society in 1928.

In 1910 Schryver married Miss E. Davies, who, with their two daughters, survives him.

In the course of unsuccessful attempts to effect synthesis of proteins from peptones in presence of intestinal mucous membrane his attention was drawn to the phenomenon of autolysis, which was investigated in some detail (1904, 1905, 1906). He finally reached the conclusion that the autolysis of the organs *in vivo* was regulated by chemical means and that the normal supply of nitrogenous food yielded by its breakdown sufficient ammonia to prevent the tissues from becoming acid and then undergoing autolysis.

Whilst at University College he also made experiments (with Hamill) on the excretion of nitrogen by normal individuals, finding that it corresponded to a daily intake of 93 g. of protein. In the course of investigations carried out for the Local Government Board he devised new and delicate methods for the detection of tin and of formaldehyde in foodstuffs. The application of the latter test enabled him to show the production of formaldehyde when chlorophyll was exposed to sunlight in the presence of moist carbon dioxide.

At the Cancer Hospital he carried out with Dr C. Singer investigations on the gastric juice in malignant and non-malignant diseases of the stomach and duodenum (1913) in which chemical methods (*e.g.* determination of the peptic index and of the ratio of acid combined with amino-acids to total nitrogen) were applied to diagnosis. Here too he began work on the state of aggregation of matter which led directly to the study of clot formation, the results of which formed the subject of a series of papers published between 1910 and 1919. These included useful studies of the relation between the viscosity and surface tension of salt solutions and the effect of these salts on clotting and gel formation, and an intensive examination of the clotting of milk by rennin, in the course of which he showed that caseinogen is converted into a less soluble "metacaseinogen" by treatment with hot water, but that this substance differs in properties from the casein produced by rennin.

Schryver's deep interest in gel formation was maintained in the investigations which he carried out at the Imperial College. These followed two main lines. In the first place he attacked the constituents of plant cells, preparing and studying the proteins of leaves and also studying the cell wall, from which he isolated the pectins and hemicelluloses which take a part in its structure. He came to the conclusion that pectinogen is a trimethyl ester of pectic acid loosely combined with metallic ions, such as Ca, and that pectic acid and the hemicelluloses, a number of which were isolated from various materials, were both complexes of "uronic acids" and sugars, as suggested by Ling and Ehrlich. For such complexes he suggested the name "polyuronides." Lignification of tissue he found (1928) to be accompanied by the disappearance of pectins.

The second group of researches arose from work done under the auspices of the Adhesives Research Committee of which he was made a member. This Committee was appointed by the Department of Scientific and Industrial Research in 1919 to take over the work of the Adhesives Committee originally appointed by the Conjoint Board of Scientific Societies in the latter part of 1917. This work resulted in two series of papers, on the preparation, purification and properties of gelatin and on the separation of the products of protein hydrolysis. Many of these results are also contained in the First and Second Reports of the Adhesives Research Committee (1922, 1926) and a Third Report is in course of preparation.

The formation of gelatin from its precursors, collagen and ossein, was studied from the physico-chemical standpoint with interesting results. Elaborate attempts were made to "purify" gelatin and obtain a material which could be regarded as a chemical entity, but without complete success. A large degree of purification could be effected by electrolysis and by flocculation in an electric field. The resulting material, however, is probably to some degree contaminated by products formed by the action of water, which produces an irreversible change, measurable in a few hours, even at 37°. Such "purified" gelatin is almost insoluble in water at 15° and is very similar in properties to the globulins, from which it differs by its power of readily forming gels when dispersed in either acids, alkalis or salts.

In the course of these researches the important observation was made that the composition of gelatin was not constant, but varied with the treatment to which either the gelatin itself or its precursor had been subjected. This was detected by a variation in the percentage of the total nitrogen of the hydrolysis products which was precipitated by phosphotungstic acid (diamino-nitrogen). Thus the hydrolysis products of gelatin from a precursor which had been treated with dilute alkali yielded 29·5 %, whereas those of gelatin from the same precursor treated with dilute acid gave only 26·00 % of diamino-nitrogen: gelatin itself showed similar changes.

These facts have been criticised by Daft, working in the Carlsberg Laboratory, but the criticism has been refuted by Thimann, one of Schryver's students. The investigation of this phenomenon led to the work on the separation of the products of protein hydrolysis. In this pioneering work, which must be regarded as preliminary and awaiting further extension and confirmation, Schryver developed a method of separation of the products of protein hydrolysis which depended on the use of Siegfried's barium carbamate compounds of the amino-acids. In this way he obtained from the hydrolysis products of isinglass (and edestin, but not of caseinogen or egg-albumin) hydroxylysine, in the form of a barium carbamate soluble in 70 % alcohol but insoluble in cold water, whilst from the glutelin which he isolated from oats he obtained two new hydroxyamino-acids, hydroxyaminobutyric acid, which forms an insoluble copper salt, and hydroxyaminovaleric acid, which forms a soluble copper salt.

In addition to this he obtained a new eight-carbon acid, $C_8H_{15}O_3N_3$, which he termed protoctine, from the phosphotungstic acid precipitate obtained from the soluble carbamate from glutelin and the protein of castor bean. Finally, *dl*-lysine was obtained from gelatin which had been treated with acid before being hydrolysed and to this the increased percentage of diamino-nitrogen found in such gelatin was traced. No clue was, however, found to the origin of this extra lysine.

Schryver (with Chibnall) also initiated an investigation into the proteins of leaves, which has been continued with great success by Chibnall. He, moreover, isolated a crystalline substance, $C_3H_8O_4$, from cabbage leaves which he regarded as a disaccharide derived from glycollic aldehyde and formaldehyde.

From 1918 up to the time of his death he was also engaged in researches on the autolysis and nutrition of yeast in connection with the Research Scheme of the Institute of Brewing. The interesting fact was observed that when yeast is washed it only autolyses very slowly, and much work was expended in endeavouring to trace the presumed agent removed by washing, but without any definite result (1927). The work on yeast nutrition had only reached a preliminary stage at the time of Schryver's death.

He was the author of two books. *An introduction to the study of biological chemistry* (1918) provided a general introduction to organic chemistry, followed by special chapters on "The chief chemical constituents of the animal body (fats and lipoids, carbohydrates, proteins)"; "The methods employed for the investigation of chemical changes within the animal organism (metabolism, total and intermediary)"; and "The chemical processes taking place in plants (including enzyme actions)." This was also the plan he adopted in his lectures. He also contributed in 1909 an early volume to Longmans Series of Monographs on Biochemistry on *The general characteristics of the proteins.*

Many of Schryver's researches were published in collaboration with his students, a number of whom are now pursuing independent investigations in continuation of the work first begun under his direction at South Kensington.

A. H.

XXIX. THE EFFECT OF CEREAL EXTRACTS ON BLOOD-CALCIUM.

BY LOUIS MIRVISH.

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(*Received December 31st, 1929.*)

THE concentration of calcium in the blood is remarkably constant in man and in the laboratory animals so far investigated. Apart from such procedures as dilution of the blood, or administration of salts of calcium or phosphorus, the blood-calcium has not been found to vary to any appreciable extent, except under the influence of certain internal secretions. Collip [1925] has established the rôle of the parathyroid in raising the blood-calcium, and Mirvish and Bosman [1927] have shown that extracts of bovine ovaries are able to bring about a fall of blood-calcium in a characteristic manner in both rabbits and man. A very similar action was found for extracts of suprarenal cortex [Mirvish and Bosman, 1929, 1], and probably also for the testis [Mirvish and Bosman, 1929, 2]. Bearing in mind the necessity for correlation of animal and plant physiology, I proceeded to investigate extracts of plant organs, and chose oatmeal as a convenient possible source of this "ovarian" substance.

Oatmeal has been shown to be associated in some way with a pathological phase of calcium metabolism, *i.e.* rickets. The chief evidence in this connection has been brought forward by E. Mellanby [1922], who showed that puppies on a bare sufficiency of the antirachitic vitamin D could be made to develop rickets by feeding on a diet of cereals, the most potent rickets-producing of which was oatmeal. This work was later extended by Green and Mellanby [1928], who showed that the same interfering action of cereals on the calcification of bones could be demonstrated in rats, and an ingenious method was evolved by these observers to determine quantitatively the degree of anti-calcifying activity of different cereals. They also showed that this anti-calcifying power could be counteracted by simultaneous administration of more vitamin D or of calcium carbonate. They could, however, destroy the anti-calcifying factor by boiling the oatmeal with 1 % HCl. Holst [1927] has obtained similar results. He was able to produce rickets in rats by feeding exclusively on cereals and yellow peas, but not by feeding with rice-starch alone. If oatmeal is extracted with dilute hydrochloric acid, and the extract is given in addition to the starch, the animals develop rickets. It seems, therefore, that here is a positive factor, which Holst calls a "toxic substance," the mode of action of which cannot be accounted for by the vitamin-deficiency theory of rickets. Holst finds that the production of rickets by feeding with cereals can be prevented by the administration of calcium salts, whereas phosphates have no such effect. M. Mellanby [1923], working on puppies, found that the greater the amount of cereal eaten, other things being equal, the greater the tendency to the production of badly calcified teeth.

Another series of observations of a different kind points to the presence in certain plants of an "ovarian" substance. There is evidence that some plants contain a substance of the same nature as the "female sex hormone" or "oestrin," as seen by the partially positive vaginal smear reactions obtained with extracts of flax and hemp seeds [Kountz, 1927], and by the positive oestrus obtained by Loewe and his associates [1927], using willow catkins and other plants.

The purpose of the present experiments was to attempt to obtain from oatmeal a substance similar to that extracted from bovine ovaries in its action in lowering the blood-calcium.

EXPERIMENTAL.

The cereals employed for extraction were oatmeal, wheat germ, and maize meal, but the majority of the observations were made on finely ground, untreated oatmeal. In the earlier part of my work rectified spirit was used to extract the cereal meal directly, either by percolation in the cold or by extracting on the water-bath and purifying as in the case of the ovary. Most of these extracts gave positive results when tested on the blood-calcium, as did simple aqueous extracts. But these methods of extraction were not used further, as I wanted to ascertain whether the extract of oatmeal with dilute hydrochloric acid, which Holst found to be rachitogenic, possessed also the calcium-reducing property. The method eventually adopted for the preparation of extracts, of which a preliminary account has already been given [Mirvish, 1929], was as follows.

The meal was put in canvas bags, which were suspended in a bath containing the solution for extraction, usually 0·5 % to 1 % HCl in water, and left to soak for a day or two. The bath was then placed in a larger water-bath and heated for 2–4 hours. The fluid extract was then poured off, and the bags of meal drained without being squeezed. The bags of meal were then extracted a second time either with plain water or again with 0·5 % HCl in water exactly as before, and the two extracts combined. On an average 6 litres of water were used to extract 1 kg. of meal, yielding a yellowish solution containing a minimum of starch.

The solution was next evaporated to small bulk. During all evaporations, sodium hydroxide was added from time to time, in order to keep the solution constantly weakly acid to litmus. When the bulk of the solution was reduced to about a quarter of the original volume, it was filtered through glass-wool and the evaporation continued until the final volume was about 100 cc. It was then dialysed against distilled water for 24–48 hours. The inner liquid was discarded and the yellow dialysate evaporated to small volume. Several crops of crystals (mostly NaCl) could be removed by cooling on ice and further evaporation. Finally one obtained a brownish-coloured paste, and for a time a neutralised aqueous solution of this was used for injection. Later, however, this material was extracted with boiling 95 % alcohol, and the oily yellowish-brown liquid remaining after evaporation of the alcohol was dissolved in water and used for injection.

The technique for estimating the effect of the extracts on the blood-calcium level was that employed for studying the effect of the ovarian extract [Mirvish and Bosman, 1927]. Rabbits, kept under standard conditions, were injected with varying amounts of the extracts, and blood was drawn before injection and at various intervals after injection. The previous work on ovary showed that the blood-calcium falls most frequently 24–48 hours after injection. Control injections of other tissues (muscle, brain, pancreas, spleen, kidney), and of cholesterol and olive oil produce no such fall in blood-calcium, and one must therefore attribute it to the specific substance present in the extract. Some of the results obtained with various types of extract are given in Table I.

Table I. *Injection of various extracts of oatmeal compared with injection of ovarian extract.*

		Serum-calcium in mg. per 100 cc.			
Rabbit	Injected subcutaneously	Normal	After 24 hrs.	After 48 hrs.	After 72 hrs.
	Oatmeal extracted with 95 % alcohol.				
11	Extract representing 40 g. oatmeal	17·5	18·0	17·5	16·9
12	,, ,, 60 g. ,,	17·5	14·5	14·5	16·5
39	,, ,, 150 g. ,,	15·0	10·0	12·1	—
40	,, ,, 375 g. ,,	14·0	11·3	13·8	—
41	,, ,, 375 g. ,,	15·0	12·2	11·0	—
	Oatmeal extracted with water.				
R 1	Extract *W* representing 150 g. oatmeal	13·5	12·0	12·5	11·0
R 2	,, ,, 300 g. ,,	14·0	11·0	11·5	10·0
R 5	,, ,, 200 g. ,,	12·8	10·5	—	—
R 6	,, ,, 600 g. ,,	11·5	6·6	—	—
	Oatmeal extracted with 0·5 % HCl.				
Y 4	Extract *A* representing 200 g. oatmeal	13·1	13·1	9·3	14·3
Y 5	,, ,, 250 g. ,,	13·6	13·1	9·8	—
Y 6	,, ,, 650 g. ,,	12·1	8·8	10·7	13·6
16	,, *E* ,, 180 g. ,,	15·3	13·55	14·5	11·5
17	,, ,, 270 g. ,,	16·7	14·0	11·8	—
20	,, *G* ,, 400 g. ,,	14·0	15·0	11·5	—
21	,, ,, 800 g. ,,	11·5	12·0	7·0	—
	Alcoholic extracts of bovine ovary.				
A	Representing 25 g. of ovary	14·5	10·5	15·0	—
B	,, 40 g. ,,	15·0	13·0	11·5	—
C	,, 25 g. ,,	17·0	14·5	14·0	—

These results are very similar to those obtained with ovarian extract, the only difference being that the minimal effective dose is much larger than in the case of the ovary. I next tested the dialysate of this material, as Holst found that his rachitogenic fraction was dialysable, and this fraction, as well as an alcoholic solution of it, was also found to be calcium-reducing. The method of extraction is of course by no means quantitative, nevertheless it is apparent that each process of refinement of the extract progressively lowers its potency (Table II).

Table II. *Injection of dialysed extracts of oatmeal.*

		Serum-calcium in mg. per 100 cc.				
Rabbit	Injected subcutaneously	Normal	After 24 hrs.	After 48 hrs.	After 72 hrs.	After 96 hrs.
	Oatmeal extracted with 0·5 % HCl and dialysed. Dialysate employed.					
35	Representing 150 g. oatmeal	16·0	17·0	17·5	16·0	—
37	,, 225 g. ,,	12·5	13·8	13·3	14·0	14·0
38	,, 350 g. ,,	14·0	14 5	14·7	15·0	14·7
39	,, * 350 g. ,,	14·0	12·3	11·2	13·7	13·7
40	,, 1000 g. ,,	15·0	12·5	10·2	10·3	13·8
	Oatmeal extracted with 0·5 % HCl, dialysate then extracted with 95 % alcohol.					
14	Representing 600 g. oatmeal	15·0	15·5	15·0	15·0	15·0
15	,, 600 g. ,,	16·0	17·0	16·0	15·0	16·5
16	,, 960 g. ,,	16·0	11·0	15·0	15·0	16·0
17	,, 1200 g. ,,	15·0	14·0	12·5	15·0	15·0
19	,, 1800 g. ,,	17·5	13·5	15·0	13·0	15·0
20	,, 1920 g. ,,	17·5	12·0	16·0	15·5	17·0
21	,, 2160 g. ,,	18·0	16·0	13·0	16·0	16·0
9	,, 960 g. ,,	17·5	14·5	16·0	16·0	15·0
10	,, 1600 g. ,,	18·0	15·0	13·5	15·0	18·0
11	,, 2400 g. ,,	16·0	10·0	14·9	16·0	17·0

* Injected intraperitoneally.

DISCUSSION.

In general the results show that oatmeal extracts are able to reduce the blood-calcium of rabbits, both male and female, the maximum depression occurring on an average at the end of 24 hours, with a return to normal usually within 72 hours after administration. The more effective the dose the earlier is the point of maximum depression, so that it may occur as early as 10 hours after injection. Intraperitoneal injection has a more rapid action than subcutaneous. It has been found that some rabbits do not react at all to the injection, although other animals of the same batch may react to a similar dose of the extract employed. The same experience was also encountered with extracts of ovary, testis and suprarenal cortex, though the cause is not clear. The calcium-reducing substance is present in oatmeal in a concentration of about 1/10 to 1/5 of that of the bovine whole ovary. The experiments demonstrate that oatmeal has the same effect as ovary on the blood-calcium, attributable to a substance which I called "calcovarin" in previous work.

It has been found by various observers that the oral administration of oatmeal produces rickets. This effect has been traced to a dialysable substance which can be extracted from oatmeal by dilute HCl. An extract obtained in the same manner, injected in large doses into animals, produces a noticeable fall of blood-calcium of the same order as that produced by extracts of bovine ovary. It has not been determined whether this oatmeal extract given by mouth will reduce the blood-calcium, but this is the case with the ovarian extract. It would seem then that the rachitogenic and the blood-calcium-reducing actions are both a function of the same extract, and one must attribute the development of rickets in these cases to an over-supply of "calcovarin."

Another condition where the calcium metabolism is profoundly disturbed is osteomalacia, in which the bones are much softened, the blood-calcium is low (6–8 mg. per 100 cc.), and tetany is frequent. It has been found that the diet of women thus affected contains excess of cereals, and administration of cod-liver oil or exposure to sunlight cures or prevents the disease and restores the normal blood-calcium level. There is considerable evidence of a relationship of the ovarian function to osteomalacia, as it occurs almost exclusively in women, is usually associated with puberty or pregnancy, and removal of the ovaries frequently cures the condition. Recent investigations have disclosed that its occurrence at puberty before pregnancy is at least as common as its association with pregnancy, so that the bony softening cannot be explained away as due to a drain on the maternal calcium for the requirements of the foetus. In spite of the different types of bony defects, there is no doubt that osteomalacia and rickets are closely related diseases occurring at different ages. Bearing in mind that the ovarian secretion has the same influence on calcium metabolism as the rachitogenic element in oatmeal, one can postulate that osteomalacia is due to excessive "calcovarin" supplied to the body partly from an exogenous source, the cereal diet, and partly from an endogenous source, namely that elaborated by the hyperactivity of the ovary, which occurs at puberty and pregnancy.

We can only put forward a working theory at present on the connection between "calcovarin" and vitamin D. Rickets and osteomalacia are both recognised as being primarily diseases of calcium metabolism, and all our clinical and metabolic studies emphasise the fact that calcium metabolism is controlled by an endocrine mechanism, in which the parathyroid plays the leading part. A disturbed state of calcium metabolism, short of gross diminution of calcium intake, should lead us to search for an underlying endocrine disturbance. There is evidence that the ovary produces its effect on the blood-calcium through the parathyroid, and that leads one to suggest the possibility that rickets and osteomalacia are caused by a functional hypo-parathyroidism. It may be that vitamin D is necessary for the proper function of the parathyroid, and, as the body cannot synthesise vitamin D in the absence of sunlight, parathyroid secretion is not elaborated sufficiently and a hypo-parathyroidism or some disturbance of the parathyroid apparatus results. "Calcovarin" also produces a functional hypo-parathyroidism by reacting with or antagonising the parathyroid secretion. Thus a deficiency of vitamin D, or an over-supply of "calcovarin"—endogenous as in osteomalacia, or exogenous as in rickets—

will lead to parathyroid disturbance. This hypothesis may not be warranted by the facts at our disposal, but we know that vitamins have some relation to the endocrine secretions, and it may be that vitamins are necessary for the proper elaboration of the various internal secretions. The production of rickets by a positive factor challenges our vitamin theory and demands explanation.

SUMMARY.

A method has been found for extracting oatmeal with 0·5 % hydrochloric acid, yielding an extract which is free from starch, thermostable, dialysable, and soluble in alcohol. This extract, when injected into rabbits, produces a fall of blood-calcium. The extent of this fall is about 30 % of the normal. It occurs 24–48 hours after injection, the figure returning to normal again within 48–72 hours. The type of calcium depression is the same as was found previously to follow the injection of bovine ovarian extract. From this it is concluded that "calcovarin," the substance producing the characteristic calcium depression response, is present in oatmeal as well as in ovary. The oatmeal extract which Mellanby and Holst found to be rachitogenic in puppies and rats was prepared in the same way as the extract here tested, and the rachitogenic effect of this extract is attributed to its calcium-reducing property. A suggestion is put forward that rickets and osteomalacia are diseases which may be due to functional disturbances of the parathyroid secretion.

I desire to express my thanks to the Research Grant Board of the Union of South Africa, and to the Staff Research Fund of the University of Cape Town for grants, which partly defrayed the expenses of this research.

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XXX. THE TYROSINASE-TYROSINE REACTION.

VII. THE ACTION OF TYROSINASE ON CERTAIN SUBSTANCES RELATED TO TYROSINE.

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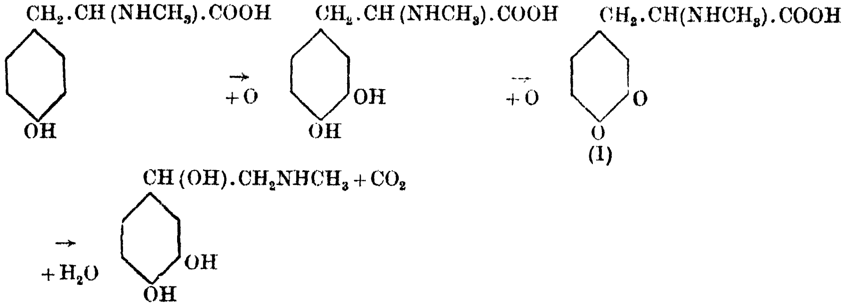
IN Part VI of this series [Raper, 1927] the identification of 5 : 6-dihydroxyindole and 5 : 6-dihydroxyindole-2-carboxylic acid as products of the action of tyrosinase on tyrosine was described. It seemed of interest to find out whether certain substances closely related to tyrosine when oxidised by means of tyrosinase would also give rise to indole derivatives, and with this object the following substances have been submitted to the action of the enzyme: tyramine, 3 : 4-dihydroxyphenylethylamine, 3 : 4-dihydroxyphenylethylmethylamine (epinine) and *N*-methyltyrosine. Using the procedure that has been previously described [Raper, 1927] it was possible to isolate 5 : 6-dimethoxyindole from the products of oxidation both of tyramine and of 3 : 4-dihydroxyphenylethylamine. From epinine, a dimethoxy-derivative was obtained which is presumably 5 : 6-dimethoxy-*N*-methylindole, though this substance has not yet been synthesised for comparison. With *N*-methyltyrosine evidence of the production of an indole derivative has been obtained but it was not isolated in crystalline fórm. These experiments indicate therefore that the series of reactions undergone by tyrosine to give rise eventually to 5 : 6-dihydroxyindole and its 2-carboxylic acid is a general one provided that a 3 : 4-quinone is produced in the initial stages of the oxidation process. It has already been shown by Pugh and Raper [1927] that the production of orthoquinones from those monohydric phenols on which tyrosinase acts is characteristic of this enzyme and that they are also produced by it from *o*-dihydric phenols. It was not surprising therefore that the above-mentioned substances related to tyrosine, which contain a hydroxyl group in the *para*-position or have hydroxyl groups in the 3 : 4 positions, should undergo similar changes.

Of the series of reactions which takes place when tyrosine is acted upon by tyrosinase the only specific one is the production of 3 : 4-dihydroxyphenylalanine. The subsequent oxidation of this to the corresponding orthoquinone and the further transformation of this in the manner previously described [Raper, 1927] should be capable of being brought about by any oxidising agent that produces orthoquinones from catechol derivatives. It was decided therefore to find out whether evidence supporting this could be obtained by oxidising 3 : 4-dihydroxyphenylalanine and epinine with a mild oxidising agent. Both these substances were in fact found to be readily oxidised when shaken with freshly prepared moist silver oxide and as products of the oxidation 5 : 6-dihydroxyindole-2-carboxylic acid and 5 : 6-dihydroxy-*N*-methylindole, respectively, were detected by the isolation of their corresponding methoxy-derivatives.

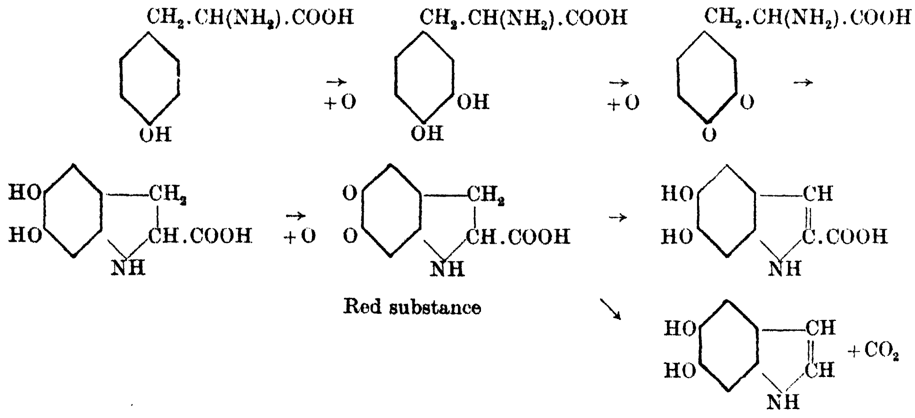
In the oxidation of tyramine, 3 : 4-dihydroxyphenylethylamine, epinine and *N*-methyltyrosine with tyrosinase certain minor differences as compared with the behaviour of tyrosine were observed. The *o*-dihydroxy-compounds are much more rapidly oxidised than tyrosine to give red substances and the latter (presumably the 5 : 6-quinones of the corresponding dihydro-indole derivatives) are more stable if separated from the enzyme than the red substance produced from tyrosine and 3 : 4-dihydroxyphenylalanine. *N*-methyltyrosine is oxidised more slowly than tyrosine and also gives rise to a somewhat more stable red substance. These results indicate therefore that the velocity of the internal oxidation-reduction process by which the 5 : 6-quinone of dihydroxyindole-2-carboxylic acid is converted into a 5 : 6-dihydroxyindole derivative is increased by the presence of the carboxyl group and that methylation of the nitrogen atom of the indole has a tendency to diminish the speed of this change. The oxidation of *N*-methyltyrosine is of particular interest because of the close relationship that this substance bears to adrenaline. It is conceivable that *N*-methyltyrosine might give rise to adrenaline by oxidation and loss of CO_2 as follows:



The production of the 3 : 4-quinone of phenyl-*N*-methylalanine (I) by the action of tyrosinase is in full accord with what we already know of the action of this enzyme. In the scheme represented above the conversion of this quinone into adrenaline with the evolution of CO_2 is represented as an internal oxidation-reduction reaction. With tyrosine, the quinone corresponding to (I) changes at once into a dihydroxyindole derivative and although this same type of change has also been demonstrated to take place readily with the quinone corresponding to epinine, it appears to take place less readily with the quinone produced from *N*-methyltyrosine, judged by the yield of indole derivative obtained. It is possible therefore that the quinone (I) represented above undergoes, in part, some change different from the usual one and the production of adrenaline from it, in the manner suggested, is a possibility to be borne in mind. A few experiments which have been carried out show that a small amount of a pressor substance is produced when the red solution produced by the action of tyrosinase on *N*-methyltyrosine is allowed to decolorise *in vacuo*. Whether this pressor substance is adrenaline cannot yet be stated and much more evidence is necessary before this can be settled.

All the four substances related to tyrosine mentioned above finally give rise to melanin when the action of the enzyme is sufficiently prolonged and carried out at about p_H 8·0. At p_H 6·0 melanin production is much slower and this is especially so with tyramine, and *N*-methyltyrosine. The fact, however, that the *N*-methyl-derivatives give rise to melanin suggests that the nitrogen atom of 5 : 6-dihydroxyindole is not further oxidised in the formation of melanin from tyrosine.

In order to get more precise information regarding the oxidative changes by which melanin is produced from 5 : 6-dihydroxyindole the oxidation of tyrosine and several of the related substances already mentioned has been studied, using a respirometer to measure the oxygen uptake. This method gives, in addition, quantitative evidence of the extent to which tyrosine undergoes the series of changes already described in Parts V and VI of this series when it is oxidised by tyrosinase. The evidence for these changes has been obtained by isolation of 3 : 4-dihydroxyphenylalanine and the methoxyindole derivatives. This is by no means a quantitative method and it seemed desirable to determine whether the oxygen uptake per atom of tyrosine corresponds with that theoretically deduced. The following scheme represents the changes which have been postulated [Raper, 1927] to explain the conversion of tyrosine into 5 : 6-dihydroxyindole:



It will be observed that 3 atoms of oxygen are required to convert 1 molecule of tyrosine into 5 : 6-dihydroxyindole. These 3 atoms have been taken up by the time the red substance has been formed so that the final change of this to dihydroxyindole will take place in an inert atmosphere. Melanin formed from tyrosine has been found to contain 8·65 % of nitrogen [Raper and Wormall, 1925]. Dihydroxyindole, $C_8H_7O_2N$, contains 9·39 % of nitrogen. By the loss of 2 atoms of hydrogen and the addition of 1 atom of oxygen it would yield a substance $C_8H_5O_3N$ which contains 8·59 % nitrogen. If this be the empirical formula of melanin then its formation from 5 : 6-dihydroxyindole would entail the utilisation of 2 atoms of oxygen. Starting from tyrosine therefore, 5 atoms of oxygen in all would be required to produce melanin. The mean of several experiments yielded the figure 5·23 atoms per molecule of tyrosine. With 3 : 4-dihydroxyphenylalanine 4 atoms of oxygen should be required to produce melanin. Experiment in this instance gave the figure 4·12. Tyramine and epinine both show a much slower rate of conversion of the red substance to dihydroxyindole or dihydroxy-*N*-methylindole respectively, and the figures 4·75 and 3·92 were obtained instead of the theoretical 5 and 4 respectively. These results suggest therefore that 2 atoms of oxygen are required for the conversion of each molecule of dihydroxyindole or its *N*-methyl-derivative into melanin and they confirm the explanation already given of the various stages by which tyrosine is converted into 5 : 6-dihydroxyindole and its 2-carboxylic acid. *N*-methyltyrosine and adrenaline have also been examined by the respirometric method. The former took up 4·92 atoms of oxygen and this corresponds fairly well with the 5 atoms required theoretically for the production of melanin. It has already been pointed out above, however, that the course of oxidation of this substance may be more complex than that of tyrosine so that the interpretation of this result is uncertain. Adrenaline was expected to take up only 3 atoms of oxygen but took up 5·3. It is, however, unlikely that the stages in the oxidation of adrenaline are the same as those for the other substances described above. When oxidised by tyrosinase it yields a black precipitate and a deep reddish-brown solution, differing in this respect from tyrosine, which is completely converted into melanin.

EXPERIMENTAL.

The enzyme used in the following experiments was prepared from mealworms by the method already described [Raper, 1926].

Action of tyrosinase on tyramine.

Tyramine hydrochloride was prepared by the method of Johnson and Deschavsky [1925]. 1 g. of the hydrochloride in 1 litre of water was warmed to 25–30° and 100 cc. enzyme solution were added. The reaction was rapidly adjusted to p_H 6–6·5 by addition of very dilute ammonia or acetic acid as required, the solution saturated with oxygen in a stoppered bottle and well shaken. Oxygen was bubbled through at half-hour intervals and the shaking repeated frequently. A red colour developed after about 5 minutes and gradually deepened. After 3 hours the enzyme had precipitated. 10 cc. of 1 % acetic acid were added, the solution was filtered and 30 cc. of a saturated solution of SO_2 added. After standing in a stoppered bottle for 24 hours the solution was distilled under reduced pressure in a stream of hydrogen until it was reduced to about 20 cc. The reaction products were now methylated in an atmosphere of hydrogen, using 13 cc. of 20 % NaOH and 3 cc. of dimethyl sulphate. The whole of the alkali was added at once, the solution warmed slightly and the dimethyl sulphate added in three portions with vigorous shaking. After warming for an hour on the water-bath the solution was cooled and shaken out three times with its own volume of ether. The combined ether extracts were washed with a little 2 % sulphuric acid, then with water and evaporated. A crystalline residue was left. A trace of this dissolved in alcohol gave a good indole reaction with *p*-dimethylaminobenzaldehyde and concentrated HCl. It also gave a reddish-violet colour with sodium nitroprusside and caustic soda, which was turned to blue on acidifying with acetic acid. The residue crystallised twice from alcohol melted at 154–5° and when mixed with 5 : 6-dimethoxyindole the M.P. remained unchanged. An alternative method is to allow the red solution obtained as described above to decolorise in an atmosphere of hydrogen. The process is hastened by the addition of 2 cc. 10 % NaOH. This should be introduced after evacuation of the flask containing the solution, shaking to get rid of oxygen, and then admitting hydrogen. The disappearance of the red colour takes about 3 days at 25°. The evaporation in a stream of hydrogen and the subsequent methylation may then be carried out as described above. The ether extract on evaporation yields 5 : 6-dimethoxyindole.

Action of tyrosinase on 3 : 4-*dihydroxyphenylethylamine.*

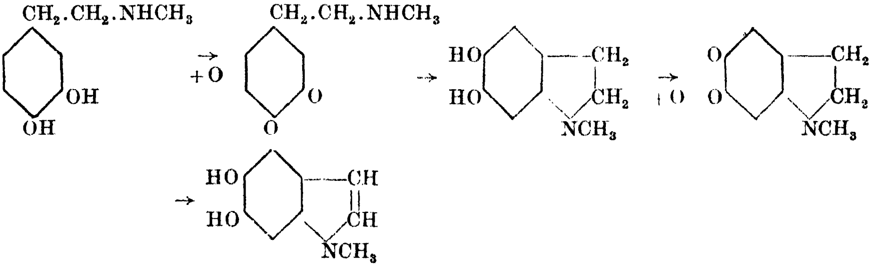
We are indebted to Prof. R. Robinson for the 3 : 4-dihydroxyphenylethylamine used in this experiment. 1 g. of the hydrochloride was dissolved in 2 litres of water warmed to 25° and 100 cc. of the enzyme solution were added. The p_H was quickly adjusted to 6–6·5. On saturation with oxygen and shaking a deep red colour rapidly developed and within a few minutes the enzyme had precipitated. This is in marked contrast with the period of about 3 hours required to reach the same stage with tyramine. After half an hour the solution was filtered and placed in a flask, which was then evacuated and filled with hydrogen. 2 cc. 10 % NaOH were added and the solution was kept under hydrogen for 3 days, when its colour had become a pale brown. It was evaporated to small bulk *in vacuo* in a stream of hydrogen and methylated as described in the experiments with tyramine. The ether extract yielded 0·103 g. of a crystalline residue which on recrystallisation from absolute alcohol melted at 154–5°; mixed M.P. with 5 : 6-dimethoxyindole, 154–5°. The substance gave good indole reactions with *p*-dimethylaminobenzaldehyde and with sodium nitroprusside.

Action of tyrosinase on 3 : 4-*dihydroxyphenylethylmethylamine.*

The 3 : 4-dihydroxyphenylethylmethylamine hydrochloride (epinine) was obtained from Messrs. Burroughs, Wellcome and Co. The procedure used was exactly as described above for tyramine. The epinine was rapidly attacked by tyrosinase and in 10 minutes yielded a deep red solution with accompanying precipitation of the enzyme. The red substance was allowed to undergo conversion into the dihydroxyindole derivative either by keeping under hydrogen or by adding sulphurous acid as described with tyramine. After concentration of the resulting pale brown solution and methylation the ether extract yielded a crystalline solid. From 1·9 g. of epinine, 0·40 g. was obtained. This was recrystallised from absolute alcohol until its M.P. was constant at 138–9°.

Micro-analysis: found, H, 6·7 and 6·45 %; C, 68·85 and 68·98 %. Calculated for $C_{11}H_{13}NO_2$, H, 6·8 %; C, 69·1 %.

A solution in alcohol gave a strong indole reaction with *p*-dimethylaminobenzaldehyde and a drop of conc. HCl. It did not give a colour reaction with sodium nitroprusside and NaOH, thus differing from 5 : 6-dimethoxyindole. A trace of the substance dissolved in glacial acetic acid gave a yellow colour changing to brown on adding a drop of conc. HNO_3 and warming; no trace of orange or red colour was produced. From analogy with the behaviour of 3 : 4-dihydroxyphenylethylamine with tyrosinase the substance obtained as above from epinine should be 5 : 6-dimethoxy-*N*-methylindole, the stages in the reaction being represented as follows:



A satisfactory method for the synthesis of 5 : 6-dimethoxy-*N*-methylindole has not yet been found, but from the known changes undergone by tyramine and 3 : 4-dihydroxyphenylethylamine when oxidised by tyrosinase there can be little doubt that the substance obtained from epinine is the corresponding *N*-methylindole. It will be shown presently that epinine when oxidised with silver oxide yields the same substance.

Action of tyrosinase on N-*methyltyrosine.*

The *N*-methyltyrosine was kindly prepared for us by Dr J. F. Wilkinson by the method of Johnson and Nicolet [1913].

1 g. of the amino-acid was dissolved in 750 cc. boiling water, diluted to 2 litres and cooled to 30°. 80 cc. of the enzyme solution were added, the p_H adjusted to 6·5 and the liquid saturated with oxygen and shaken vigorously. The saturation with oxygen was repeated half-hourly, and the solution was shaken at frequent intervals. A red colour was soon produced, and after 2½ hours the enzyme had precipitated. 10 cc. of 1 % acetic acid were added, the solution filtered, and the red filtrate after evacuating and shaking to

remove oxygen was kept under hydrogen until the red colour had given place to a pale brown. The solution was distilled under reduced pressure in a stream of hydrogen until about 20 cc. were left. Methylation was carried out in the usual way, using 3 cc. dimethyl sulphate and 12 cc. of 20 % NaOH. The alkaline solution was extracted three times with its own volume of ether, and the ether extracts were washed with a little water. On distilling off the ether about 50 mg. of a clear resinous mass was obtained which partially crystallised on dissolving in alcohol and evaporating. It gave a strong indole reaction with *p*-dimethylaminobenzaldehyde. After ether-extraction of the alkaline solution resulting from the methylation, it was acidified to Congo red and again extracted with ether, but only a trace of ether-soluble substance was obtained. No greater success was obtained when the red substance obtained by acting on *N*-methyltyrosine with tyrosinase was allowed to decolorise in presence of sulphurous acid. After methylation only a trace of ether-soluble substance was obtained from the acidified solution. Judged by previous experiments with tyrosine it was expected that a reasonable amount of either 5 : 6-dimethoxy-*N*-methylindole or its 2-carboxylic acid should have been obtained according to the procedure used in the decoloration of the red substance. As pointed out before [Raper, 1927], the use of sulphurous acid favours the formation of the indolecarboxylic acid. The above results show that with *N*-methyltyrosine the internal oxidation-reduction process by which the 5 : 6-quinone of dihydroxyindole-2-carboxylic acid is converted into the corresponding 5 : 6-dihydroxyindole-2-carboxylic acid is more difficult when the nitrogen of the dihydroindole ring is methylated. It is possible that the oxidation of *N*-methyltyrosine by tyrosinase does not proceed entirely on the same lines as that of tyrosine. In the introduction to this communication it has been pointed out that by simple oxidative changes and loss of CO_2 adrenaline might be produced. In order to determine whether any pressor substance could be detected amongst the oxidation products a solution of 0·025 g. *N*-methyltyrosine in 50 cc. of water was treated with 3 cc. of the enzyme, the p_H being adjusted to 6·5. After 3 hours, when a good red colour had been produced and the enzyme had precipitated, a few drops of 0·1 % acetic acid were added and the solution filtered. A portion of the filtrate was kept *in vacuo* in a Thunberg tube for 3 days, when it had almost lost its colour. This solution was tested for pressor activity by our colleague, Dr A. D. Macdonald, to whom our best thanks are due. In the decerebrate cat it produced a rise in blood-pressure judged to be equivalent to that given by 1 in 300,000 adrenaline. When the red solution obtained by oxidation was decolorised in presence of sulphurous acid, the pressor activity was less. Controls with the enzyme solution alone had no action.

Oxidation of 3: 4-dihydroxyphenylalanine and epinine with silver oxide.

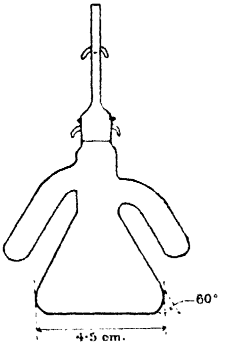
Tyrosine is not attacked when shaken with freshly prepared silver oxide. It is well known, however, that catechol derivatives easily reduce ammoniacal silver solutions and it was therefore expected that both 3 : 4-dihydroxyphenylalanine and epinine would be oxidised by silver oxide suspensions. Preliminary experiments showed that this was so and a more detailed investigation was carried out.

3: 4-Dihydroxyphenylalanine. 1 g. of the amino-acid was dissolved in 400 cc. hot water and cooled to room temperature. To this a suspension of 2·4 g. freshly prepared silver oxide in about 100 cc. water were added and the liquid was vigorously shaken for 15 minutes. The solution rapidly became coloured red and later, deep reddish brown. 10 cc. of a 10 % solution of sodium sulphate and 10 cc. of 10 % acetic acid were now added, the solution was shaken for a short time, to assist coagulation of the colloidal silver, and finally allowed to stand for 30 minutes. It was filtered by suction through filter paper pulp, 20 cc. of a saturated solution of SO_2 were added to the filtrate and it was left overnight. After filtering from a small amount of deposit it was concentrated *in vacuo* in a stream of hydrogen to about 20 cc. The products of the reaction were methylated under hydrogen, 8 cc. of 40 % NaOH were added, the solution was warmed slightly and then 3 cc. dimethyl sulphate were added in three portions with vigorous shaking. The alkaline solution was finally heated on a boiling water-bath for an hour. After cooling it was extracted three times with ether but only a trace of ether-soluble substance was removed. It was then acidified with sulphuric acid to Congo red and again extracted three times with its own volume of ether. The combined ether extracts were washed with a little water and the ether distilled off. The crude residue weighed 0·139 g. This was extracted with about 10 cc. boiling water, filtered to remove tarry matter and the filtrate taken to dryness in a vacuum desiccator. At this stage the products of two preparations carried out as above were worked up together. After crystallising four times from benzene containing 5 % of acetone colourless plates were obtained, M.P. 202–3° with vigorous evolution of gas. This corresponds with the behaviour of 5 : 6-dimethoxyindole-2-carboxylic acid which loses CO_2 at its melting point and is converted into 5 : 6-dimethoxyindole. A mixture with the synthetic acid gave the same M.P. The acid gave a good indole reaction with *p*-dimethylaminobenzaldehyde as also did the residue obtained when the acid was allowed to decompose at its M.P. These results show that silver oxide and tyrosinase can both bring about the same oxidative changes in 3 : 4-dihydroxyphenylalanine. The yield of dimethoxyindole-2-carboxylic acid obtained with silver oxide is smaller than with tyrosinase. Probably side reactions occur with the former.

Epinine. 1 g. epinine in 400 cc. water was treated with 3 g. freshly prepared silver oxide in the way described above for 3 : 4-dihydroxyphenylalanine. After removal of the silver by filtration sulphurous acid was added to the solution and it was left overnight. It was reduced to 10 cc. by distillation *in vacuo* in a current of hydrogen and methylation carried out in the usual way, using 3 cc. dimethyl sulphate and 8 cc. of 40 % NaOH. Extraction of the alkaline liquid with ether yielded 0·142 g. of crude methylation product. This was recrystallised twice from alcohol and melted at 138–9°. The M.P. was unchanged on mixing with the similar product obtained by the oxidation of epinine with tyrosinase, which is presumably 5 : 6-dimethoxy-*N*-methylindole.

Respirometer experiments.

These were carried out in a modified form of Haldane's blood gas apparatus [1920]. Instead of the simple flasks used by Haldane, conical flasks with two bent side arms, as illustrated, were employed.



The substrate was placed in the body of the flask, the enzyme solution in the lower side arm and a little 30 % KOH in the other. By tilting the flask, the enzyme may be added to the substrate after equilibration without removing the flask from the thermostat. The KOH absorbs any CO_2 which may be produced. Flasks with ground-glass stoppers were used in the later experiments as they were found to give more consistent results. The experiments lasted several days and during the frequent shaking during this period rubber stoppers may suffer slight displacement which affects the reading.

Solutions used. Tyrosine, 0·05 %; tyramine hydrochloride, 0·1 %; epinine, 0·1 %; 3 : 4-dihydroxyphenylalanine, 0·1 %; adrenaline (free base), 0·1 %; *N*-methyltyrosine, 0·01 %; all in distilled water. In each instance 1 cc. of enzyme solution was used. The reaction flask contained 4 cc. phosphate buffer, of p_H 6 or 8 according to requirements, and 4 cc. of the above tyrosine solution or 2 cc. of the solution of the other substances with the exception of *N*-methyltyrosine, of which 10 cc. were used. Thymol was used as antiseptic. The control flask contained the same amount of enzyme and buffer solutions, but no substrate. The enzyme and KOH solutions were easily introduced into the side arms by means of a curved pipette. The bottles were completely immersed in a thermostat at room temperature and were mechanically shaken. With the taps open to air, 10 minutes' shaking was allowed for equilibration. The taps were then closed and after a further 15 minutes' shaking a reading was taken. This was repeated, if necessary, to ensure that equilibrium was established before starting the experiment. The enzyme was now added from the side arm of the experimental flask and the shaking continued. Readings were taken periodically until oxygen uptake finally ceased, and for confirmation for at least two days after this. The readings were corrected for barometric pressure and temperature, and are expressed in terms of the number of atoms of oxygen taken up per molecule of substrate used (Table I).

Table I.

Substrate and its amount	p_H	No. of exps	cc. O_2 absorbed at N.T.P.	Average O_2 uptake cc.	O_2 uptake. Atoms per molecule of substrate
Tyrosine (2 mg.)	6	1	0·62	0·62	5·00
	8	5	0·656–0·631	0·647	5·23
l-3 : 4-Dihydroxyphenylalanine (2 mg.)	6	2	0·440–0·430	0·435	3·82
	8	5	0·49 –0·447	0·469	4·12
i-3 : 4-Dihydroxyphenylalanine (2 mg.)	8	2	0·466–0·466	0·466	4·09
Epinine (2 mg.)	6	1	0·409	0·409	3·71
	8	7	0·456–0·408	0·431	3·92
Tyramine hydrochloride (2 mg.)	6	2	0·538–0·535	0·537	4·17
	8	9	0·646–0·565	0·613	4·75
N-Methyltyrosine (1 mg.)	8	1	0·276	0·276	4·80
	Unbuffered	2	0·308–0·258	0·283	4·92
Adrenaline (2 mg.)	8	4	0·66 –0·63	0·65	5·30

Consideration of these results shows that at p_H 8 the oxygen uptake is always higher than at p_H 6, though the differences are not great. The number of atoms taken up per molecule of substrate is in accordance with the view that tyrosine and tyramine require 3 atoms, and dihydroxyphenylalanine requires 2 atoms, of oxygen to convert them into 5 : 6-dihydroxyindole and a further 2 atoms to convert this into melanin. Epinine requires 2 atoms of oxygen to convert it into 5 : 6-dihydroxy-*N*-methylindole and a further 2 atoms to transform this into melanin. It is possible that reactions other than the main one account for the slight divergence from the theoretical values at p_H 8. At p_H 6 the slower rate of action of the enzyme and its precipitation after a time probably account largely for the oxygen uptake being less than the expected value. The red substances produced by tyramine and epinine are also much more stable than that from tyrosine and much less rapidly converted into the colourless dihydroxyindole derivatives. For this reason these substrates are probably not completely converted into melanin within the period of time during which the enzyme is still active. The figures for *N*-methyltyrosine suggest that the main reaction proceeding is the same as that with tyrosine, but the possibility of other reactions occurring has already been pointed out.

It may be noted that *i*-3 : 4-dihydroxyphenylalanine, a sample of which was kindly supplied by Dr C. R. Harington, behaves exactly like the naturally occurring amino-acid. This was to be expected since the enzyme action is not concerned with oxidation of the side chain which contains the asymmetric carbon atom.

If a few drops of 30 % KOH were added to the solution in the experimental flask after all enzyme action had ceased a further oxygen uptake was observed. This occurred outside the limits of p_H at which tyrosinase is active and was probably due to production of oxidation products of melanin itself by atmospheric oxygen.

An experiment was carried out with adrenaline at p_H 8 in the absence of the enzyme. The oxygen uptake observed was 6·14 atoms per molecule of adrenaline as opposed to 5·29 with the enzyme present. It is probable therefore that oxidation of adrenaline in the air, with the production of the well-known pink colour, proceeds on different lines from that produced by the enzyme, but what these are can only be discovered by further investigation.

SUMMARY.

(1) As a result of the action of tyrosinase, tyramine and 3 : 4-dihydroxyphenylethylamine yield 5 : 6-dihydroxyindole, 3 : 4-dihydroxyphenylethylamine yields 5 : 6-dihydroxy-*N*-methylindole, and *N*-methyltyrosine yields an indole derivative not yet identified.

(2) *N*-methyltyrosine on oxidation with tyrosinase also yields a small amount of a pressor substance.

(3) 3 : 4-Dihydroxyphenylalanine and 3 : 4-dihydroxyphenylethylmethylamine on oxidation with silver oxide behave in the same way as when oxidised by tyrosinase and yield 5 : 6-dihydroxyindole-2-carboxylic acid and 5 : 6-dihydroxy-*N*-methylindole respectively.

(4) In the production of melanin from tyrosine and tyramine approximately 5 atoms of oxygen are utilised per molecule of substrate, whereas 4 atoms are required by 3 : 4-dihydroxyphenylalanine and 3 : 4-dihydroxyphenylethylmethylamine. It is probable that 2 atoms of oxygen are needed to convert 5 : 6-dihydroxyindole into melanin.

We wish to express our thanks to the Government Grant Committee of the Royal Society for a grant in aid of this work.

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XXXI. SULPHUR IN ORGANIC AND INORGANIC COMBINATION IN PASTURE GRASSES.

By HARRY ARTHUR ASHWELL AITKEN.

Report to the Department of Scientific and Industrial Research, New Zealand.

(*Received January 30th, 1930.*)

The sulphur content of pastures has recently become a subject of considerable importance both from the point of view of sulphur fertilisation of soils [Alway, 1927] and in relation to the sulphur requirements of sheep for wool-production [Robertson and Marston, 1928]. The question of sulphur deficiency in soils has led to very thorough surveys of the total sulphur content of pastures in different parts of the world, but the organic sulphur content of pastures has received little or no attention. Nevertheless the separation of the total sulphur content of grasses into organic and inorganic components is important in seeking a correlation between the sulphur content of pastures and their value for purposes of wool-production, as only organic sulphur can be made use of by animals in biological synthesis. In the present paper, a method is described whereby the organic sulphur of pastures can be readily determined. It is assumed that all the inorganic sulphur in green leaves is in the form of sulphate, whether free or as ethereal sulphate. This sulphate-sulphur is therefore estimated and deducted from the total sulphur, giving a value for the organic sulphur.

As regards the nature of the organic sulphur compounds in grass, they probably occur as constituents of protein. Woodman [1926] finds 27·92 % crude protein in autumn cuts of English grass, while Rigg and Askew [1929] find an average value of 33·37 % crude protein for eight samples of New Zealand pasture. "Crude protein" includes chlorophyll and other non-protein nitrogen compounds, but the figures are of the right order to account for the organic sulphur present in grass, assuming that grass-protein contains the average amount of sulphur. In this connection it is to be noted that a water-soluble protein prepared from grass, as subsequently described, by the method of Chibnall and Schryver [1921] did not contain sulphur. This substance was a proteose, however, not a native protein, and it represented only a small proportion of the total nitrogen of the grass.

Since protein-sulphur in grass would have to form part of an amino-acid, the question of the identity of this sulphur amino-acid immediately arises. Robertson and Marston [1928] apparently make a tacit assumption that the acid is cystine, this being the sulphur constituent of wool and also of wide-spread occurrence elsewhere in nature [Jones, Gersdorff and Moeller, 1924].

Such cystine could be directly assimilated by sheep and used in the growth of wool. However, it is significant that Mueller [1923] finds that methionine, a sulphur amino-acid isolated from caseinogen, occurs in wool along with cystine. In the experimental section of this paper, attempts to find cystine in grass by the ordinary precipitation and colorimetric methods are described. Though these were greatly hampered by the difficulty of decolorising the hydrolytic products from grass, the results obtained were consistently negative, from which it would appear that most of the organic sulphur of grass is not in the form of cystine.

EXPERIMENTAL.

Preliminary treatment of the grass.

The following grasses were analysed for sulphur: Cocksfoot (*Dactylis glomerata*), Perennial Rye (*Lolium perenne*), Prairie Grass (*Bromus unioloides*), Yorkshire Fog (*Holcus lanatus*), and Hair Grass (*Festuca bromoides*). Only the blades were collected as these give a more homogeneous sample than is obtained by the inclusion of stalks and heads, which are drier and less palatable to stock. The grass was freed from colourless and withered leaves and foreign matter, rinsed with water and spread on wire netting to dry. It was then reduced to a handy form for analysis by toasting on a hot plate and passing through a mincer. The arrangement consisted of a galvanised iron tray 3 ft. by 2 ft. standing on large tripods; 2 inches below this, a sheet of iron was supported on smaller tripods and heated by four Bunsen burners, the hot air from its upper surface maintaining the bottom of the tray at about 120°. The grass was turned continually till dry and then clipped into short lengths. When sufficiently brittle it was put through a mincer and readily reduced to a fine powder. The powder was placed in bottles which were heated to 110° in a toluene-oven and carefully stoppered.

In order to determine the moisture content of the grasses, quantities (about 8 g.) of the fresh, superficially dry specimen were placed in two beakers. One sample was dried in a toluene-oven at 110°, the other in an air-oven at 120°. The values obtained generally agreed within 0·3 %.

Estimation of total sulphur in powdered grass.

The estimation of sulphur in grass by means of sodium peroxide as used at the Rowett Institute, Aberdeen, apart from its rather difficult technique, has the disadvantage that occasionally sudden ignition occurs, rendering the estimation worthless, and also that the sodium peroxide rapidly destroys the nickel crucibles. A method was therefore devised by which the Benedict-Denis reagent could be used for the oxidation, and this proved both rapid and satisfactory. After several trials, the following procedure was adopted. 1–1·5 g. of grass powder is transferred from a weighing bottle to a 4 in. porcelain dish. 50 cc. of the Benedict-Denis reagent (125 g. crystallised copper nitrate, 125 g. sodium chloride, 50 g. ammonium nitrate, 500 cc. water, all free from sulphate) is then added and the mixture evaporated to dryness on a curved asbestos gauze. It is then brought to a dull red heat at which the copper oxide formed completely oxidises the grass. The product is taken up with 50 cc. of 2*N* HCl, gently evaporated to dryness, and finally extracted by warming with successive portions of 2*N* HCl, 100 cc. being used for the purpose. Extracts and washings are made up to 250 cc. This is filtered by suction and portions of 100 cc. are taken in which sulphate is estimated by precipitation with barium chloride. In the precipitation of small amounts of barium sulphate, easy filtration is ensured by carrying out the precipitation at the boiling point in 3 % hydrochloric acid, using a large excess of barium chloride. The solution will clear in less than 3 hours and the precipitate is crystalline.

Three grasses in which sulphur was estimated, both by means of sodium peroxide and the Benedict-Denis reagent, gave the following results:

	Cocksfoot	Perennial Rye	Yorkshire Fog	
Sodium peroxide method	0·438	0·460	0·391	% sulphur
Benedict-Denis method	0·442	0·462	0·390	

Estimation of sulphate-sulphur in powdered grass.

Attempts to extract the sulphates present in powdered grass by simply boiling with dilute hydrochloric acid gave rise to solutions containing colloids which were liable to be absorbed when the sulphates were precipitated with barium chloride. To overcome this, experiments were made in which the grass was first subjected to fusion in nickel crucibles with such agents as sodium carbonate, sodium acetate, borax, etc. and then extracted. This certainly obviated the formation of colloids, but apparently led to reduction of some of the sulphate, as low and variable results were obtained. The method of Rosenheim and Drummond, using benzidine hydrochloride as a precipitant, was then tried on a grass extract, the presence of colloids being disregarded as the estimation depends on a titration with alkali. Low values were obtained, however, as the alkali reacted very slowly with the precipitate, protected as it was with colloidal material. Finally it was discovered that decolorising charcoal could be conveniently used to remove colloids yielding a solution quite suitable for gravimetric estimation of sulphate. After some preliminary experiments, the following method was found to give satisfactorily constant results. 2–3 g. of grass powder is transferred from a weighing bottle to a 600 cc. beaker and boiled gently for half an hour with 100 cc. of 2*N* NaOH. 200 cc. of 2*N* HCl are then added and the mixture is boiled for a further half-hour. 2 g. of decolorising charcoal, previously freed from sulphate by repeated extraction with boiling hydrochloric acid, are added and the boiling continued for about 20 minutes. Finally, the product is filtered through a 4 in. Büchner filter, and the precipitate washed several times with dilute hydrochloric acid so as to leave no adhering sulphate. The extract is made up to 500 cc. and sulphate is estimated in 200 cc. portions by precipitation with barium chloride.

Results of sulphur estimations.

Examination of five common grasses yielded the results shown in Table I.

Table I.

Grass	Date of sampling	% water	% total sulphur	% sulphate-sulphur	% organic sulphur (by difference)
Cocksfoot	May 10	75·0	0·44	0·21	0·23
Perennial Rye	Aug. 15	77·1	0·46	0·23	0·23
Prairie Grass	Aug. 21	80·0	0·59	0·34	0·25
Yorkshire Fog	Aug. 21	77·0	0·39	0·13	0·26
Hair Grass	Aug. 21	73·3	0·35	0·10	0·25

It will be seen that there is no great variation in the organic sulphur content of these grasses. Indeed, Yorkshire Fog and Hair Grass, which are worthless as pasture, are slightly higher in organic sulphur than the valuable grasses Cocksfoot and Perennial Rye. It follows that the organic sulphur content of grasses does not necessarily bear any relation to their palatability and nutritive value.

On the other hand, a great variation is observed in the sulphate-sulphur content of the different species, possibly due to different amounts of sulphate in the soils on which the grasses were growing. It seems probable that the increase in sulphur content of pastures observed on making sulphur applications to soil is due to an increase in the inorganic sulphur of the grasses.

The water-soluble protein of grass.

In investigating the question of the occurrence of cystine in grass, an obvious point of attack is the separation and hydrolysis of the leaf protein. Hence it was decided to prepare and examine the leaf protein described by Chibnall and Schryver [1921]. This was carried out as follows. About 6 lb. of fresh Perennial Rye grass was minced first with a coarse, then with a fine cutter, and treated with an equal weight of water saturated with ether. After allowing 20 minutes for cytolysis to take place, the mixture was expressed through silk muslin, a green colloidal solution being obtained. This was filtered by means of a number of large funnels, as the ether present makes suction filtering disadvantageous. The resulting clear solution was light brown in colour. The water-soluble protein was precipitated by adding sufficient $2N$ H_2SO_4 to make the whole solution $N/12$. The flocculent white precipitate was separated by siphoning off the supernatant liquid, filtering, and washing the precipitate with alcohol and ether. Some 10 g. of crude protein was thus obtained. This was dissolved in 0·3 % solution of KOH in 60 % alcohol, as used by Osborne and Wakeman [1920], and precipitated by careful addition of dilute HCl. The process was repeated twice, after which the isoelectric point was determined and found to be at p_H 7·3. The protein was found to be soluble in 0·4 % ammonia in 60 % alcohol, and this was then used instead of KOH to treat the crude protein. The protein gave both the xanthoproteic and the biuret reactions, though Chibnall and Schryver could get no protein colour reactions with the similar material which they isolated from cut grass. The rose tint observed in the biuret test and the turbidity produced when the aqueous solution was treated with ammonium sulphate indicated that the substance was a simple proteose or hydrolysed protein. The purified material was carefully tested and found to contain no sulphur. It was concluded that the organic sulphur must have remained in the solid residue from the water-soluble products. A portion of this was analysed and found to contain 0·42 % of sulphur. Hence it was decided to hydrolyse the whole grass and test the product for cystine.

Testing the products of hydrolysis of grass for cystine.

Folin's method for the isolation of cystine was first applied to grass powder as follows. 500 g. of powdered Cocksfoot grass were hydrolysed with 2 litres of 20 % HCl on the water-bath for 144 hours. The liquid was filtered, partially neutralised with 150 cc. of 40 % NaOH and evaporated to low volume at 10 mm. pressure. Hot saturated sodium acetate was added till there was no mineral acid reaction to Congo red, and after standing for two days the precipitate was filtered by suction, dissolved in 100 cc. of 5 % HCl and boiled with successive quantities of decolorising charcoal till it was faintly yellow in colour. The solution was then brought to p_H 5 and allowed to stand. A white amorphous precipitate containing a good deal of calcium oxalate was formed and this was tested for cystine as described by Hunter and Eagles [1927] but without result. Finally the precipitate was tested with nitroprusside and found to contain no sulphur. In view of the quantity of grass taken, such a result throws considerable doubt on the assumption that the whole of the organic sulphur in grass can be represented as cystine.

It was now decided to test the hydrolysis products colorimetrically for cystine. The method of Sullivan [1925, 1926] was adopted as this is supposed to be the most specific estimation short of direct isolation. The test solution was prepared by boiling 500 g. of grass powder with conc. HCl for 6 hours, filtering, distilling off most of the acid *in vacuo*, taking up with water and decolorising by means of charcoal. Finally it was concentrated to about 200 cc., cooled, rendered slightly alkaline with 10 % ammonia [Okabe, 1928], filtered and reacidified. On testing 2 cc. with sodium 1 : 2-naphthaquinone-4-sulphonate, as described by Rimington [1929], no red colour stable to hydrosulphite could be produced. Thus the colorimetric method also yielded a negative result. A point to be considered, however, is that great difficulty is experienced in decolorising the hydrolysis products from grass, which almost resemble ink in appearance, the colour being perhaps due to combination of tannin with the iron present in the grass. It has been shown by Folin and Marenzi [1929] that cystine is considerably adsorbed by decolorising charcoal, but it is hardly conceivable that the whole of the cystine could be thus adsorbed. Again, the toasting process might have produced chemical changes in the cystine, which is not as stable in protein as in the free state [Brand and

Sandberg, 1926]. Hence fresh grass was used instead of grass powder. Several pounds of fresh, minced Cocksfoot were hydrolysed with concentrated HCl in a large silica basin for 6 hours. The liquid was filtered, concentrated *in vacuo* nearly to dryness, taken up with water, treated with 30 % sodium acetate solution till neutral to Congo red, allowed to stand for 48 hours and filtered. Precipitate A was so obtained. Half of the filtrate was then treated by Gaskell's [1908] method for the isolation of cystine from urine: the solution was rendered slightly alkaline with 10 % ammonia, treated with an equal volume of acetone and just acidified with acetic acid, when a precipitate settled out and was filtered off (Precipitate B). Precipitates A and B were united, dissolved in dilute HCl, decolorised with charcoal and tested for cystine by Sullivan's method. The result was negative. The remaining half of the filtrate was then treated with 50 cc. of a 30 % solution of phosphotungstic acid in 5 % H_2SO_4. A gummy brownish precipitate was obtained (Precipitate C). This was washed with dilute phosphotungstic acid solution and then dissolved in dilute NaOH. Phosphotungstic acid was removed by means of barium chloride and the barium by means of sulphuric acid. The resulting solution was decolorised with charcoal and tested by Sullivan's method. This time a slight colour was obtained, but comparison in a Kober colorimeter with a 0·01 % solution of cystine showed that it represented only a trace of cystine and indeed might have been due to tyrosine.

A final experiment was carried out, using Perennial Rye grass instead of Cocksfoot. The sulphate-sulphur was first removed by repeated extraction with dilute HCl, and the grass then hydrolysed with concentrated HCl in a silica basin. That hydrolysis proceeds slowly was shown by the fact that after 3 hours the insoluble residue still contained 0·20 % of sulphur. The product was examined by Gaskell's method and with phosphotungstic acid as described above, but only negative results were obtained. To sum up, no evidence was obtained from the experiments on the hydrolysis products of grass in support of the assumption that the organic sulphur of grass is present in the form of cystine.

SUMMARY.

1. The Benedict-Denis method of oxidation has been applied to the determination of the total sulphur content of grasses.

2. A method has been devised for the estimation of sulphate-sulphur in grass or green leaves.

3. The analytical results for five grasses show no relation between the nutritive value and the organic sulphur content of grasses.

4. The water-soluble protein obtained from grass did not contain sulphur.

5. The occurrence of cystine in quantity corresponding to the organic sulphur content of grass could not be demonstrated.

The author's thanks are due to Dr Malcolm, who suggested this research, for his helpful interest during the course of the work; and to Dr Inglis in whose department the work was carried out under tenure of a National Research Scholarship at Otago University.

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XXXII. SURFACE FILMS OF BATYL, CHIMYL AND SELACHYL ALCOHOLS.

By BERT CYRIL JAMES GABRIEL KNIGHT.

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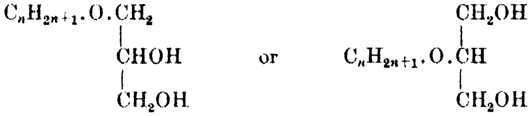
(*Received February 5th, 1930.*)

The isolation of quantities of batyl, chimyl and selachyl alcohols by Drummond and Baker [1929] in this laboratory provided an opportunity to examine thin films of a type of molecule not hitherto studied. Batyl alcohol has been shown by Heilbron and Owens [1928] to be an ether of octadecyl alcohol and glycerol. Selachyl alcohol is the corresponding glyceryl ether of oleyl alcohol, and chimyl alcohol the cetyl alcohol derivative [Drummond and Baker, 1929].

The three compounds have therefore the same type of structure, namely a long hydrocarbon chain attached by an oxygen atom to a glycerol group at either the α- or β-carbon atom:



$$\begin{array}{l} C_nH_{2n+1}.O.CH_2 \\ \qquad\qquad\quad | \\ \qquad\qquad\quad CHOH \\ \qquad\qquad\quad | \\ \qquad\qquad\quad CH_2OH \end{array} \quad \text{or} \quad \begin{array}{r} CH_2OH \\ | \quad\;\; \\ C_nH_{2n+1}.O.CH \\ | \quad\;\; \\ CH_2OH \end{array}$$

where $n = 18$ for batyl alcohol or 16 for chimyl alcohol.

In selachyl alcohol the hydrocarbon chain is probably

$$CH_3.(CH_2)_7.CH = CH.(CH_2)_7.CH_2—$$

but the position of the double bond is not definitely known.

Heilbron and Owens [1928] gave reasons for believing that the β-structure was the correct one. More recently, however [Heilbron, 1929], it is stated that attempts are being made to synthesise structures of the α-type, this being regarded as the more probable structure. The measurements on the films to be described in this paper provide strong evidence in favour of the α-structure.

EXPERIMENTAL.

The apparatus and technique used in the study of monomolecular films have been fully described by Adam and Jessop [1926]. Dilute solutions of the several alcohols in benzene were accurately prepared. The benzene was refractionated and finally distilled in an all-glass (pyrex) apparatus before use. It left no residual film upon water. A small quantity of solution was placed upon a clean water surface in a Langmuir-Adam trough by means of a capillary drop-pipette.

The water used in the trough was laboratory-distilled, further purified by redistillation from alkaline permanganate in a large pyrex still with ground joints. Before a film was applied the water surface was always tested for contamination by sliding the barrier to within 2 cm. of the float of the torsion apparatus. No deflexion is noted if the surface is clean.

Materials. One sample of batyl alcohol had been obtained from the liver oil of the Greenland shark, *Somniosus microcephalus*. It had been purified by several recrystallisations from ether and melted at 69°. Another specimen had been obtained by the hydrogenation of selachyl alcohol. After purification it also melted at 69°. The specimen of chimyl alcohol was isolated from Japanese shark-liver oil and after recrystallisation from ether melted at 62–62·5°.

The selachyl alcohol was fractionated from Japanese shark-liver oil and purified by refractionation. It boiled at 236–239° at 5 mm., but the iodine value was slightly higher than the theoretical.

The films of the three alcohols were studied between 0° and 40°, a range which includes the transition temperatures from condensed to expanded films of batyl and chimyl alcohols.

DISCUSSION.

The results of the measurements are illustrated by the curves in Figs. 1 and 2, which show the relation between the area occupied by one molecule in the film and the compressing force.

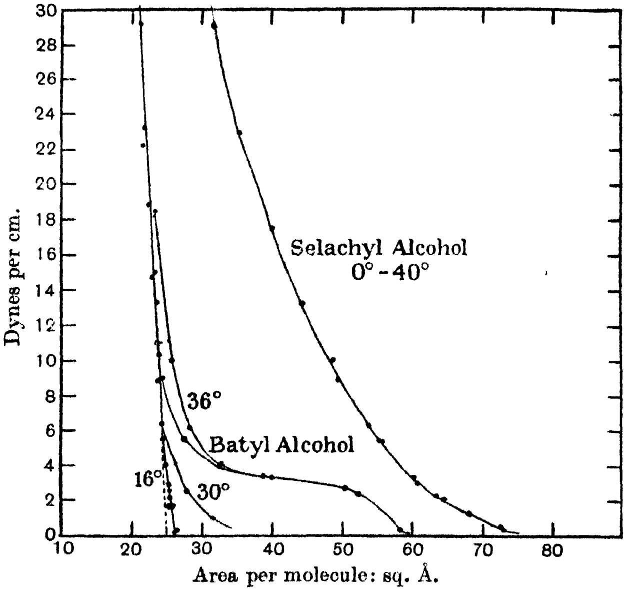


Fig. 1.

The films of batyl alcohol and chimyl alcohol showed a close resemblance to those of the α-monoglycerides [Adam, Berry and Turner, 1928], both condensed and expanded films being formed. The curves for chimyl alcohol are identical with those of α-monopalmitin at every temperature, within experimental error: the condensed and expanded films, the transition region and the temperature of expansion being practically the same.

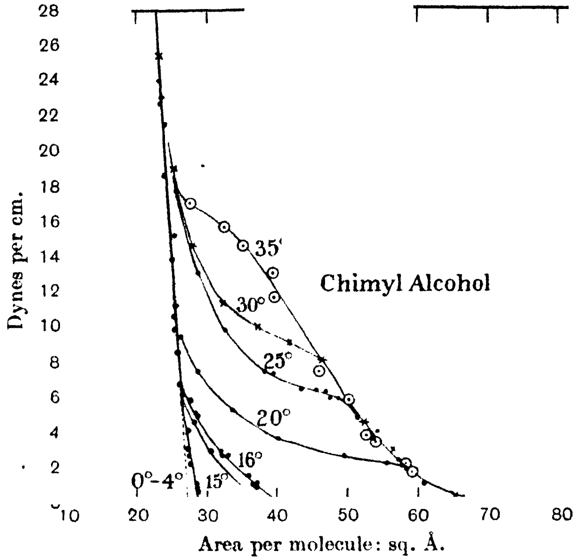


Fig. 2.

Condensed films. The condensed films are liquid, as shown by the movement of talc particles scattered on the surface. The films are of the close-packed head variety, with slight rearrangement under pressures greater than about 6 dynes/cm. The values for the cross-section of the molecule for the two arrangements (extrapolated graphically to zero pressure) are included in Table I.

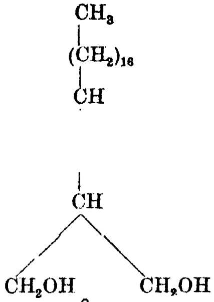
Table I.

	Cross-section, sq. Å. per molecule		
	Head	Rearranged	Difference
α-Monoglycerides	26·3	—	—
Batyl alcohol	26·0	24·8	1·2
Chimyl alcohol	28·6	27·4	1·2
(Difference)	(2·6)	(2·6)	—
Mono-octadecylmalonic acid	36	—	—

All values ±0·4 sq. Å.

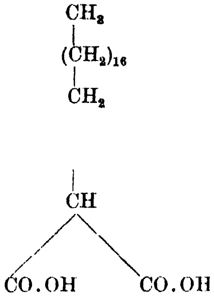
It will be seen that the curves for the condensed films of the two substances are sensibly parallel, with chimyl alcohol about 2·6 units greater in area. This may be due to a slight trace of unsaturated impurity which might be expected from the mode of preparation. The values for the cross-section given by batyl alcohol are probably more nearly correct. They agree very closely with the values found by Adam, Berry and Turner for α-monoglycerides, namely $26{\cdot}3 \pm 0{\cdot}5$ sq. Å.

This agreement is interesting in that it suggests that batyl and chimyl alcohols are α-glyceryl ethers.

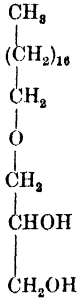


β

I.

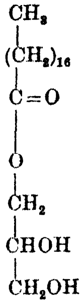


II.



α

III.



α

IV.

If batyl alcohol had the β structure I, since the two —CH_2OH groups are symmetrically placed, it might be expected to orient itself at the water surface as in the figure. In this case it would be expected that the cross-section of the head would be considerably greater than the value for cetyl alcohol, which is 21·6 sq. Å., because two —CH_2OH groups per molecule would have to be packed side by side. This is borne out by measurements of N. K. Adam (private communication) on mono-octadecylmalonic acid II. This gives a solid condensed film, with an area of about 36 sq. Å. at no compression.

If, on the other hand, batyl alcohol has the unsymmetrical α structure III, its orientation would be expected to be that of the α-monoglycerides IV, with "the three carbons of the glycerol nearly vertically above one another" [Adam, Berry and Turner, 1928]. The cross-sectional areas should therefore be the same.

The close agreement found experimentally is strong evidence in favour of the unsymmetrical α-structure for batyl and chimyl alcohols.

Expanded films. On raising the temperature the liquid condensed films give liquid expanded films. The temperatures of half expansion at 2 dynes/cm. pressure are:

chimyl alcohol	17·5°
batyl alcohol	35·5°.

The expansion is of the usual type, with the transition region not horizontal.

In the expanded films the areas at no compression are:

chimyl alcohol	65·0 ± 3 sq. Å.
batyl alcohol	60·0 ± 3 sq. Å.
α-monoglycerides	70·0 ± 3 sq. Å.

These values are interesting as further examples of expanded films having an area at no compression differing considerably from the value 48 sq. Å. found for so many substances, and upon which had been based the coiled-chain hypothesis of the structure of these liquid expanded films. This hypothesis was abandoned when it was shown that p-dodecyl- and p-nonyl-phenols and the α-monoglycerides gave areas differing from this value [Adam, Berry and Turner, 1928].

Selachyl alcohol also gives an expanded film but it is so much above its transition temperature that lowering the temperature to 0° does not cause it to condense.

SUMMARY.

Measurements have been made of monomolecular films of batyl, chimyl and selachyl alcohols between 0° and 40°.

The values for the cross-section of the heads of batyl and chimyl alcohols are compared with the value for α-monoglycerides. Evidence is adduced that these two alcohols have the unsymmetrical α-glyceryl ether structure.

I am indebted to Dr N. K. Adam for communicating to me his results on mono-octadecylmalonic acid. My best thanks are due to Prof. J. C. Drummond for supplying me with the specimens of the three alcohols. Acknowledgment is made to the Department of Scientific and Industrial Research for a personal grant.

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XXXIII. THE DECARBOXYLATION OF PECTIN.

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EARLY work on the occurrence of pectins and hemicelluloses showed that these substances were normally to be found in association in the cell-wall, the relative amount of each being dependent on the condition of the tissue, *i.e.* whether lignified or non-lignified [Clayson, Norris, and Schryver, 1921]. Subsequent work [Candlin and Schryver, 1928] indicated the existence of an intimate relation between the two classes of substances, since pectin was shown to undergo decarboxylation under the influence of alkali, yielding *inter alia* a substance of the hemicellulose type. The yield of hemicellulose obtained by this treatment was invariably low, amounting only to 12-20 % of the pectin taken; further, these workers were unable to determine the nature of the products which represented the major part of the pectin. This might have been due to the fact that, as is well known, the sugars readily undergo profound changes in the presence of alkali, giving a variety of indefinite degradation products. It would follow therefore that if, as would seem possible from its accepted constitution, the pectin molecule yields sugars on decarboxylation, these latter would be destroyed immediately by the alkali present.

Since, however, the decarboxylation of pectin is a reaction likely to throw some light upon the question of the relationship between certain of the cell-wall substances, it is desirable to establish the nature of the substances other than hemicelluloses produced thereby. With this object it is necessary to employ a decarboxylating reagent which will not tend to destroy these other products, and it has been found possible to use hot water for this purpose. By this means, pectin has been shown to yield an insoluble substance, of the hemicellulose type, and one or more soluble substances, probably sugars.

The insoluble product finally obtained appears to have undergone much more complete decarboxylation than the end-product (hemicellulose) described by Candlin and Schryver [1928]. From this insoluble product, a substance of the hemicellulose type has been separated, containing 6·2 % of uronic anhydride, and yielding a mixture of sugars on hydrolysis.

The investigation of the soluble products is not complete, but it is thought that the observation that pectin can be decarboxylated by means of hot water is of sufficient interest to warrant the publication of a brief account of the work at the present stage.

EXPERIMENTAL.

Methods of decarboxylation. For the earlier experiments, sodium pectate, prepared by the method given by Candlin and Schryver, was used. The reactions were carried out in silica vessels, as it was thought that the use of hot water under pressure might result in the liberation of free alkali from glass vessels.

About 0·4 g. sodium pectate was placed in a silica boiling-tube, with 8 cc. water, and the mixture was heated in an autoclave for 6 hours at approximately 125° (20 lb. per sq. in.). At the end of this period, the solution of pectin had assumed a faintly orange colour. The "uronic anhydride" content of the whole of the product was determined by the method of Nanji, Paton and Ling [1925]. It was found that little decarboxylation had taken place, the uronic anhydride content having fallen only by 3 % (calculated on the original uronic anhydride content of the sodium pectate).

It was thought, however, that the elimination of carbon dioxide from pectates might be a balanced reaction, in which case removal of carbon dioxide from the system would alter the equilibrium and induce further decarboxylation. With this point in view, the experimental conditions were modified as follows.

A nickel crucible containing approximately 2 g. potassium hydroxide was placed in the autoclave, the top of the crucible being well above the level of the water, but below the level of the mouth of the tube containing the sodium pectate. By the use of this device, any carbon dioxide evolved during the reaction was removed immediately.

A sample of sodium pectate heated thus for 5 hours at 153° (60 lb. per sq. in.) gave a product whose uronic anhydride content was 58 % less than that of the original sodium pectate, indicating that by this modification of the experimental conditions decarboxylation could readily be achieved. It was found that raising the temperature or prolonging the time of heating resulted in increased decarboxylation, but that at temperatures above 153° charring was liable to occur.

The possibility existed, however, that hydrolysis of the sodium pectate might occur during this process, giving rise to free alkali, which would produce secondary changes in the products of decarboxylation. For this reason it was decided to abandon the use of sodium pectate, and to use citrus pectin itself. This substance is considered by most workers to be entirely organic.

On heating citrus pectin (0·2 g. in 10 cc. water) at 153° for 1¼ hours, a fall in the uronic anhydride content of 35 % was observed. The resultant products consisted of a dark brown solid, together with an orange coloured liquid.

The insoluble products. The brown solid, which on drying was light grey in colour, was further examined.

2 g. of citrus pectin dissolved in 100 cc. water were heated in the autoclave at 153° (60 lb. per sq. in.), in the presence of potassium hydroxide, as described

above. Experiments were made to determine the effect of the time of heating on the yield of the brown product. In each case, at the end of the period of heating the reaction mixture was filtered while hot through a Munroe crucible, and the solid product was washed thoroughly with boiling water, and dried at 100°. The uronic anhydride content of the product obtained in each case was determined (Table I).

Table I.

Time of heating (hrs.)	Wt. of insoluble product		Uronic anhydride content (%)
	A† (g.)	B† (g.)	
(0	—	—	69·4*)
2	0·086	0·086	58·2
4	0·128	0·137	32·1
6	0·156	0·147	20·8; 21·2
16	0·218	0·204	12·7; 12·0
48	0·346	0·352	3·6; 3·6
96	0·403	0·413	— —

* Original citrus pectin. † Duplicate experiments.

From these figures it seems that the final product given by treatment with hot water has a very low uronic anhydride content, and that the amount of insoluble product reaches a limit on prolonged treatment. The maximum yield of the product amounted to about 20 % of the pectin taken, a figure comparable with that obtained by Candlin and Schryver for the yield of hemicellulose given by alkali decarboxylation.

For further investigation of the insoluble product, a rather larger sample was prepared by heating citrus pectin with 50 times its weight of water at 153° for 48 hours; 2 g. of product were obtained in all. This was extracted with 300 cc. of 4 % sodium hydroxide, the insoluble residue being removed. To the extract glacial acetic acid was added until the liquid was very faintly acid, after which the solution was treated with an equal volume of 95 % alcohol. A brownish coloured precipitate was produced. This settled after standing for some hours and was filtered off, washed thoroughly with 95 % alcohol, and dried. The substance thus obtained was in the form of a brown powder. A yield of 1·14 g. was obtained from 2 g. of the original insoluble product. Its uronic anhydride content was found to be 6·2 %. This is considerably lower than the uronic anhydride content of the hemicellulose described by Candlin and Schryver. The general behaviour of the substance, however, indicated that it belonged to the hemicellulose class. It yielded a mixture of sugars on hydrolysis with dilute sulphuric acid.

The soluble products. As stated above, an orange coloured liquid was obtained together with the insoluble brown product on heating pectin with water for some hours at 153°. This liquid became slightly opalescent on cooling; it was not possible to obtain a perfectly clear solution by filtration. On treatment with 4 volumes of alcohol the solution became quite clear, there being no precipitate of a gel of pectin; it was concluded therefore that the original pectin had been destroyed completely on heating. The addition of acid to the solution gave no gel of pectic acid.

The solution was neutral to litmus, and did not give the naphthoresorcinol test for sugar acids. It possessed strong reducing properties, and appeared to contain sugars. It seems certain that the use of hot water as a decarboxylating agent avoids that destruction of the soluble products which renders the use of alkali objectionable.

SUMMARY.

1. It has been found possible to produce decarboxylation of pectin by means of hot water under pressure, provided that the carbon dioxide evolved during the reaction is removed from the system.

2. The products obtained by such decarboxylation consist of an insoluble substance, probably a hemicellulose (mixed with a small quantity of undetermined matter), and one or more soluble products, possibly sugars. The hemicellulose-like substance has a uronic anhydride content of 6·2 %.

The work described in this paper was undertaken at the suggestion of the late Prof. S. B. Schryver, under whose direction the early experiments were carried out. The author wishes to place on record his indebtedness to Prof. Schryver for advice and encouragement.

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XXXIV. THE COMPOSITION OF THE MIXED FATTY ACIDS PRESENT IN THE GLYCERIDES OF COD-LIVER AND CERTAIN OTHER FISH-LIVER OILS.

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In the course of an investigation into the production and medicinal qualities of cod-liver oil obtained in various parts of the British Empire, undertaken in conjunction with Prof. J. C. Drummond of University College, London, on behalf of the Empire Marketing Board and the Medical Research Council, information of a quantitative nature has been collected as regards the composition of the mixed fatty acids present in specimens of Newfoundland, Scottish and Norwegian cod-liver oil, and also of the mixed fatty acids combined in the liver oils of coalfish (saith), hake, ling, skate and dogfish taken from off the north-eastern or north-western coasts of Scotland. The latter group of oils was from fish taken during July–September 1928 during cruises of the Scottish Fishery Board's research steamer "Explorer," and we would express our cordial thanks to the Board, and to Dr A. Bowman of their Aberdeen Marine Biological Station, for the facilities given to one of us on board the "Explorer" and for the particulars of feeding, etc., supplied in respect of the various fish.

Our object has been to collect data of an approximately quantitative kind with reference to the respective proportions of myristic, palmitic, and stearic acids, and of unsaturated acids containing respectively 16, 18, 20 and 22 carbon atoms in the molecule. The analytical methods employed do not, unfortunately, permit of the simultaneous determination of the individual unsaturated acids present, in view of the complex mixtures of these (especially in the C_{20} and C_{22} series) which are generally present in these oils. We have to content ourselves, so far as the present records are concerned, with an approximate expression of the average degree of unsaturation of each group[1], accompanied where possible by qualitative information as to the nature of the components.

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[1] This is conveniently given in terms of the average number of gram-atoms of hydrogen lacking as compared with the corresponding saturated acid: thus ($-2{\cdot}0$ H) indicates the presence of acids containing only one ethylene linkage, *i.e.* acids of the monoethylenic (oleic) series.

Whilst, so far as we know, quantitative figures of this kind have not been previously published for any of the liver oils in question, qualitative information has appeared from time to time, the earliest definite contribution in the case of cod-liver oil being that of Bull [1906], who isolated palmitoleic ($\Delta^{9:10}$ hexadecenoic acid [Armstrong and Hilditch, 1925, 2]) from cod-liver oil; references to the occurrence of highly unsaturated ("clupanodonic") acids of the C_{20} and C_{22} series in cod-liver oil were first made by Tsujimoto [1913] and by Marcusson and Botger [1914]. Other data on the fatty acids of the liver oils now under consideration may be summarised as follows.

Cod-liver oil. Palmitoleic acid [Schmidt-Nielsen, 1922], gadoleic acid [Bull, 1906], "clupanodonic" and "jecoric" acids [Fahrion, 1917]; highly unsaturated acids [Toyama and Tsuchiya, 1925]; qualitative data for the liver oil of *Gadus macrocephalus* [Toyama, 1926]; some mixed glycerides of cod-liver oil [Suzuki and Masuda, 1927, 1928]; relation of composition to vitamin content [Holmes, 1924]; percentage of highly unsaturated acids given (from insoluble polybromides) by various workers as 10-15 %.

Coalfish (saith)-liver oil. Percentage of highly unsaturated acids (from ether-insoluble polybromides) about 17 % [Lewkowitsch-Warburton, 1922].

Ling-liver oil. Percentage of highly unsaturated acids from ether-insoluble polybromides, about 14 % [Lewkowitsch-Warburton, 1922].

Dogfish-liver oil. Unsaponifiable content has been found between the limits of 4 and 32·9 %, both squalene and cholesterol being frequently present; the iodine value of the mixed fatty acids is usually lower than in the preceding cases [cf. Chapman, 1918; Lexow, 1922; Holmes and Pigott, 1925].

The sources of the oils which we have examined are given in Table I, together with additional information in some cases as to the food observed in the fish, the sex or condition, etc., whilst the general analytical characteristics of the oils, together with their comparative vitamin A potencies as indicated by the antimony trichloride colour test, are given in Table II.

In order to secure comparative data for the whole oils, the investigation was conducted in each case on the "crude" (*i.e.* not refined or chilled) material obtained by rendering the livers for a short time with open steam in a small experimental digester.

The cod- and saith-liver oils were all pale-coloured and of fair to high vitamin potency. The remainder of the specimens were much darker in colour: the ling oil and one of the hake oils were extremely rich in vitamin, but in other samples of hake-liver oil examined (four in all) the vitamin potency was of a comparatively low order, judged by the colorimetric test. The vitamin content of the skate and dogfish oils was not high, but these liver oils are of special interest from the standpoint of their component fatty acids or their content of unsaponifiable matter.

Table I.

Subdivision TELEOSTOMI. Family GADIDAE.

Species	Fish	Source, etc.
Gadus morrhua, L.	Cod, Newfoundland	Bay de Verd, 1928 season, cod feeding on squid.
„ „	Cod, Scottish	Moray Firth, end of March, 1928 (after spawning, fish mainly "spent").
„ „	Cod, Norwegian	Lofoten, 1929 season.

Species	Fish	Position	Depth m.	Date	No.	Size, etc.	Stomach contents
Gadus virens	Coalfish (saith) I	55° 24′ N., 4° 59′ W.	73	30. viii. 28	82	60–90 cm., mostly adult	Fish remains (2), herring (6), *Meganyctiphanes* (14), small *Squalus acanthias* (1), empty (59).
„ „	Coalfish (saith) II	56° 54′ N., 7° 10′ W.	148	22. viii. 28	33	42–75 cm.	Fish remains, mostly herring (21), *Gadus esmarkii* (1), *Gadus merlangus* (1), empty (10).
Merluccius merluccius	Hake I	58° 03′ N., 6° 13′ W.	137	17. viii. 28	25	48–104 cm., mostly adult	All empty
„ „	Hake II	56° 54′ N., 7° 10′ W. 56° 02′ N., 7° 03′ W.	148 78	22. viii. 28 23. viii. 28	46	38–78 cm., adult	All empty
„ „	Hake III	55° 34′ N., 5° 24½′ W.	88	12. ix. 28	16	59–100 cm., adult (2 males, 14 females)	All empty
Molva molva	Ling	58° 03′ N., 6° 12′ W.	137	17. viii. 28	1	141 cm., spent female	Empty.

Subdivision ELASMOBRANCHII. Family RAJIDAE.

Species	Fish	Position	Depth m.	Date	No.	Size, etc.	Stomach contents
Raia maculata	Skate	58° 17′ N., 6° 11′ W.	15–18	13. viii. 28	17 males 23 females	23–45 cm., 22–53 cm., developing reproductive organs	Chiefly sand-eels (*Ammodytes*), a few with Annelids and *Portunus* sp.

Family SQUALIDAE.

Species	Fish	Position	Depth m.	Date	No.	Size, etc.	Stomach contents
Squalus acanthias	Dogfish	58° 29′ N., 7° 35′ W.	130	16. viii. 28	154	47–83 cm., developing reproductive organs	Fish remains, mostly herring (110), *Moschites* (43), empty (30)

Table II. *General analytical characteristics of the liver oils.*

Fish-liver oil	Saponification equivalent	Iodine value	Free acidity (as % oleic acid)	Unsaponifiable %	Colour of oil*		Vitamin A (colorimetric) blue units
					Yellow	Red	
Cod (Newfoundland)	301·1	163·0	0·46	0·87	3·2	0·1	9·0
Cod (Scottish)	305·1	177·1	0·45	0·91	3·3	0·1	11·0
Cod (Norwegian)	302·6	161·8	0·27	0·63	1·7	0·0	3·6
Coalfish (saith) I	302·2	167·1	0·05	0·80	4·8	0·6	9·5
Coalfish (saith) II	304·8	167·5	0·07	0·60	4·9	0·5	7·8
Hake I	302·7	150·6	0·14	0·91	12·3	0·8	21·7
Hake II	304·2	158·5	0·14	1·65	6·4	0·9	3·4
Hake III	301·3	149·2	0·12	1·21	16·0	2·3	*ca.* 8
Ling	300·0	150·3	0·22	1·16	8·7	0·8	29·3
Skate	332·8	210·2	0·38	0·31	*ca.* 30	8·6	3·1
Dogfish	343·5	121·8	0·14	10·5	9·3	0·8	6·5

* Determined in a 1″ cell by the Rosenheim-Schuster colorimeter.

GENERAL EXPERIMENTAL PROCEDURE.

Broadly speaking, the method followed in determining the composition of the mixed fatty acids was based on that described for the similar analyses of the fatty acids in the head and blubber oils of the sperm whale [Hilditch and Lovern, 1928], namely, division of the acids into two (or three) groups, mainly saturated and mainly unsaturated, by crystallisation of the less soluble lead salts from alcohol, followed by conversion of each group into methyl esters and systematic fractional distillation and refractionation of the latter under as high a vacuum as possible.

Fish-liver oil acids are amongst the most difficult to handle accurately by this method for the following reasons:

(i) the unusual number and complexity of the fatty acids present;

(ii) the readiness with which the very highly unsaturated acids of the C_{20} and C_{22} series oxidise on exposure to air and polymerise when heated;

(iii) the presence of mono- (and small amounts of di-) ethylenic acids containing 16 and 14 carbon atoms (mainly palmitoleic acid, $C_{16}H_{30}O_2$);

(iv) the sparing solubility of the lead salts of the monoethylenic acids of the C_{20} and C_{22} series (gadoleic and cetoleic acids).

We introduced a number of additional precautions as described below in order to cope with these complications so far as possible, and we believe that the figures given for acids up to and including the C_{18} series are within 1 or 2 units of the true figure, but it is possible that those for the C_{20} and C_{22} acids may not in all cases be of this order of accuracy. Having pointed out, however, that the data obtained are probably not quite of the same order of certainty as in cases of simpler mixtures of acids, we must add that they furnish an approximate guide to the general composition of the acids and serve to reveal certain similarities and dissimilarities between different kinds of marine animal oils which it has not hitherto been possible to detect.

Some details of the analytical methods used are as follows.

Lead salt separations. The mixed fatty acids (1 part) from the liver oil under examination were treated with lead acetate (0·7 part) in 95 % alcohol (10 parts), the acids and the lead acetate being separately dissolved in half of the alcohol to be employed, and the boiling solutions mixed, heated under a reflux condenser for 15–20 minutes and set aside at room temperature overnight. The separated lead salts, which were frequently somewhat amorphous owing to the presence of lead gadoleate and cetoleate, were recrystallised from a volume of 95 % alcohol equal to that used in the first place and containing 1 % of acetic acid. The flasks containing the hot alcoholic solutions of lead salts were tightly stoppered before cooling, thus preventing access of air to the cold solution; liberation of fatty acids from the lead salts (and from the original saponified oil) was always carried out in an atmosphere of carbon dioxide and all collected specimens of fatty acids or the methyl esters were stored under nitrogen.

In a few instances three groups of fatty acids were esterified:

(i) those from the recrystallised lead salts ("solid" acids *S*);

(ii) those from the alcohol liquors from the recrystallisation ("liquid" acids *LR*);

(iii) those from the original alcohol liquors containing dissolved lead salts ("liquid" acids *L*); but it was observed that the acids *LR* and *L* were of much the same composition, and therefore these two groups were more usually united and dealt with as "liquid" acids *L*.

Fractional distillation. The primary distillates were usually collected in about four fractions, each of which of course was far from being a simple mixture; refractionation of each fraction, however, led to mixtures of esters which were apparently sufficiently simple for approximate estimation of their composition from consideration of the saponification equivalent and iodine value.

Myristic acid, palmitic acid, dihydroxypalmitic acid (M.P. 124–125°) and dihydroxystearic acid (M.P. 129–130°) were identified from various fractions of the Scottish cod-liver oil esters after oxidation of the corresponding soaps in alkaline solution with ice-cold permanganate, but formal identifications were not made in the cases of esters from the other oils, the fractionation data indicating similar qualitative composition in each case, and the presence of the individual acids concerned having been definitely established in the majority of the oils by previous workers.

The fractionation of the portions of esters most likely to contain highly unsaturated derivatives was in each case completed as rapidly as possible, and all ester fractions were stored under nitrogen, the iodine values being determined as soon as possible after distillation. To a large extent these precautions eliminated errors due to oxidation or polymerisation at the ordinary temperature, but in the majority of cases appreciable polymerisation of the highly unsaturated components took place during the distillations, as evidenced by the properties of the undistilled residue and progressive decline in iodine value of the higher-boiling fractions. It was therefore decided in such cases to abandon the usual method of estimation, using both iodine values and saponification equivalents, and to obtain an approximate estimate from the latter alone.

It was very noticeable that the unsaturated esters from different oils varied greatly in their tendency to undergo polymerisation during heating; some were hardly affected at all, others quite appreciably. The time and degree of exposure to heat were much the same in all cases, and it remains to be determined whether this behaviour is due to the presence of different isomeric unsaturated acids in different oils, or whether it is conditioned by some external factor such as catalytic action of the walls or internal fittings of the distillation bulb.

Calculation of the results. This was carried out on similar lines to those which we have described at length in earlier papers on this subject [cf. Hilditch and Lovern, 1928; Hilditch and Jones, 1929], with the following additions.

(i) *Composition of low-boiling fractions containing saturated esters.* Owing to the presence of unsaturated acids of the C_{16} (and C_{14}) as well as the C_{18} series, it happened not infrequently that methyl palmitate (or myristate) was accompanied by two unsaturated esters (*e.g.* methyl palmitoleate and oleate), and moreover the latter esters usually contained a certain proportion of diethylenic as well as monoethylenic compounds. This upset the usual procedure by which a mixture of two saturated and one unsaturated ester can be arithmetically analysed from its iodine value and saponification equivalent, and in such cases the ester fractions concerned (1 part) were oxidised in acetone solution (10–15 parts) with sufficient powdered potassium permanganate (2–5 parts, according to the unsaturation of the fraction) to convert all ethylenic esters present into acidic products [cf. Armstrong and Hilditch, 1925, 1]; the residual saturated esters were weighed and their composition estimated from their observed saponification equivalent.

(ii) *Composition of high-boiling fractions containing possibly polymerised C_{20} and C_{22} esters.* In order to estimate these by means of the equivalent alone, as indicated above, it was necessary to deduce the average equivalents of the unsaturated C_{20} and C_{22} esters present, since these were lower by several units than those of the corresponding saturated esters. To do this the assumption was made that mixtures of unsaturated esters of the same molecular carbon content distil together in constant proportions—an assumption which we know from experience to be very nearly, but not rigidly, correct in the case of methyl oleate and methyl linoleate. Then, commencing from the simpler mixtures of lowest boiling point (*e.g.* methyl palmitoleate with methyl oleate and linoleate) it was possible to determine the average unsaturation of the latter esters; and, by selecting suitable fractions corresponding with mixtures of unsaturated C_{18} and C_{20} esters, to repeat the calculation and determine an approximate average iodine value for the C_{20} group; and, again, similarly for the C_{22} group of esters. These approximate iodine values determine within small limits the average molecular weights (equivalents) of the corresponding groups of esters.

It is proposed to give, by way of illustration, the detailed estimations of increments of the various groups of esters in the analysis of the Newfoundland cod-liver oil, but, in order to shorten the tabulated results as much as possible, the experimental data for the remaining ten oils are tabulated and followed by a statement of the final fatty acid compositions arrived at from each analysis.

NEWFOUNDLAND COD-LIVER OIL.

Table III *a*. *Lead salt separation.*

	g.	%	Saponification equivalent	Iodine value
"Solid" acids *S*	74·0	21·1	266·2	59·4
"Liquid" acids *L*	276·0	78·9	286·0	173·4

Table III *b*. *Fractionation of methyl esters.*

Primary fractions					Refractionations				
No.	g.	B.P./1 mm.	Sap. eq.	I.V.	No.	g.	B.P./1 mm.	Sap. eq.	I.V.
(i) Esters of "solid" acids *S*.									
S 1	37·71	110–130°	261·8	19·6	*S* 11	17·62	100–120°	256·1	10·9
					S 12	8·13	120–125	266·1	17·2
					S 13	7·36	Residue	284·0	42·3
S 2	15·73	130–160	287·8	61·4					
S 3	5·47	160–163	309·0	104·3					
S 4	11·40	Residue	337·0*	145·7					
(ii) Esters of "liquid" acids *L*.									
L 1	55·81	122–140°	269·1	86·3	*L* 11	4·98	100–131°	253·4	60·4
					L 12	35·87	131–132	263·5	82·4
					L 13	10·22	Residue	285·5	115·5
L 2	67·90	140–150	291·5	125·5	*L* 21	7·25	115–142	267·7	101·5
					L 22	49·46	142–145	287·1	120·4
					L 23	6·88	Residue	293·0	200·8
L 3	76·36	150–170	319·1	215·1	*L* 31	6·48	135–156	301·6	161·4
					L 32	55·87	156–162	312·2	214·5
					L 33	9·42	Residue	333·2	259·9
L 4	14·33	170–181	338·6	260·7					
L 5	19·96	Residue	377·9*	234·8					

* Saponification equivalents of residual esters, freed from unsaponifiable material. *S* 4: 330·6, *L* 5: 332·4.

Table III *c*. *Determination of saturated components of ester fractions by oxidation.*

		Saturated ester present	
No.	Weight taken g.	g.	Sap. eq.
S 11	13·39	11·59	256·6
S 2	11·71	5·26	275·9
L 12	31·70	7·87	251·3

Table III *d*. *Estimated composition of fatty acids.*

(i) Details of methyl ester fractions.

			Esters of saturated acids			Esters of unsaturated acids					
No.	g.		C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}	Unsaponifiable matter
Esters of "solid" acids S.											
Refractionation of primary fraction *S* 1.											
S 11	17·62		8·15	7·44	—	—	2·03	—	—	—	—
S 12	8·13		0·86	5·79	—	—	1·48	—	—	—	—
S 13	7·36		—	3·25	0·48	—	—	3·63	—	—	—
	33·11	Wts.	9·01	16·48	0·48	—	3·51	3·63	—	—	—
		% as esters	27·2	49·8	1·4	—	10·6	11·0	—	—	—
Primary fractions.											
S 1	37·71		10·26	18·77	0·55	—	4·00	4·13	—	—	—
S 2	15·73		—	5·46	1·60	—	—	7·87	0·80	—	—
S 3	5·47		—	—	—	—	—	2·62	2·85	—	—
S 4	11·40		—	—	—	—	—	—	7·78	3·62	—
	70·31	**Wts.**	10·26	24·23	2·15	—	4·00	14·62	11·43	3·62	—
		% as esters	14·5	34·6	3·0	—	5·7	20·8	16·2	5·2	—
		% as acids	14·4	34·5	3·0	—	5·7	20·9	16·3	5·2	—

Table III *d* (*continued*).

No.	g.		Esters of saturated acids			Esters of unsaturated acids					Unsaponifiable matter
			C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}	
						Esters of "liquid" acids L.					
						Refractionation of primary fractions *L* 1, *L* 2, *L* 3.					
L 11	4·98		1·92	—	—	0·70	2·36	—	—	—	—
L 12	35·87		5·73	3·18	—	—	26·96	—	—	—	—
L 13	10·22		—	—	—	—	3·37	6·85	—	—	—
	51·07	Wts.	7·65	3·18	—	0·70	32·69	6·85	—	—	—
		% as esters	15·0	6·2	—	1·4	64·0	13·4	—	—	—
L 21	7·25		—	—	—	—	5·78	1·47	—	—	—
L 22	49·46		—	—	—	—	13·14	36·32	—	—	—
L 23	6·88		—	—	—	—	—	2·77	4·11	—	—
	63·59	Wts.	—	—	—	—	18·92	40·56	4·11	—	—
		% as esters	—	—	—	—	29·8	63·8	6·4	—	—
L 31	6·48		—	—	—	—	4·65	1·83	—	—	—
L 32	55·87		—	—	—	—	16·46	39·41	—	—	—
L 33	9·42		—	—	—	—	—	4·51	4·91	—	—
	71·77	Wts.	—	—	—	—	21·11	45·75	4·91	—	—
		% as esters	—	—	—	—	29·4	63·8	6·8	—	—
						Primary fractions.					
L 1	55·81		8·36	3·47	—	0·76	35·73	7·49	—	—	—
L 2	67·90		—	—	—	—	20·20	43·30	4·40	—	—
L 3	76·36		—	—	—	—	—	22·46	48·67	5·23	—
L 4	14·33		—	—	—	—	—	—	3·46	10·87	—
L 5	19·96		—	—	—	—	—	—	8·52	9·04	2·40
	234·36	Wts.	8·36	3·47	—	0·76	55·93	73·25	65·05	25·14	2·40
		% as esters	3·6	1·4	—	0·3	23·9	31·3	27·8	10·7	1·0
		% as acids	3·5	1·4	—	0·3	23·8	31·3	27·8	10·8	1·1

(ii) Composition of original acids.

Acids	"Solid" acids *S* (21·1 %)	"Liquid" acids *L* (78·9 %)	Total %	Fatty acids %	(mean unsaturation)
Saturated:					
Myristic	3·0	2·8	5·8	6	
Palmitic	7·3	1·1	8·4	8·5	
Stearic	0·6	—	0·6	0·5	
Unsaturated:					
C_{14} group	—	0·2	0·2	Trace	
C_{16} ,,	1·2	18·8	20·0	20	(−2·3 H)
C_{18} ,,	4·4	24·7	29·1	29	(−2·8 H)
C_{20} ,,	3·5	21·9	25·4	26	(−6·0 H)
C_{22} ,,	1·1	8·5	9·6	10	(−6·9 H)
Unsaponifiable	—	0·9	0·9	—	

The iodine values of the higher-boiling unsaturated ester fractions fell off somewhat on each distillation, and the average unsaturation of the acids of the C_{20} and C_{22} groups is therefore actually higher than the values suggested in the final column. The unsaturated C_{18} acids, however, consisted of about 60 % oleic and 40 % linoleic acids, whilst the corresponding C_{16} group contained a little hexadecadienoic acid but was mainly composed of palmitoleic acid. A certain amount of gadoleic (monoethylenic C_{20}) acid was present, but, apparently, little cetoleic acid ($C_{22}H_{42}O_2$).

SCOTTISH COD-LIVER OIL.

Table IV *a*. *Lead salt separation.*

	g.	%	Sap. eq.	I.V.
"Solid" acids *S*	200·2	46·5	309·5 (E)	110·3 (E)
"Liquid" acids *L*	138·6	32·2	297·4 (A)	229·7 (A)
"Liquid" acids *LR*	91·3	21·3	—	203·8 (E)

(A), analyses on acids; (E), analyses on corresponding methyl esters.

Table IV *b*. *Fractionation of methyl esters.*

	Primary fractions				Refractionations				
No.	g.	B.P./1 mm.	Sap. eq.	I.V.	No.	g.	B.P./1 mm.	Sap. eq.	I.V.
(i) Esters of "solid" acids *S*.									
					S 11	7·69	120–134°	253·0	19·8
S 1	75·28	125–135°	270·7	40·1	*S* 12	17·93	134–138	259·8	21·5
					S 13	36·32	138–152	271·7	36·8
					S 14	7·56	Residue	299·3	104·0
					S 21	13·92	145–163	285·3	66·2
S 2	49·70	135–155	297·1	102·3	*S* 22	21·25	163–165	296·6	94·5
					S 23	8·85	Residue	317·8	158·8
					S 31	8·75	165–183	318·4	158·9
S 3	57·28	155–180	329·9	189·0	*S* 32	18·40	183	325·9	181·5
					S 33	15·19	183–195	338·3	203·9
					S 34	9·69	Residue	340·4	206·3
S 4	15·11	Residue	348·7	204·5					
	Further refractionation of *S* 22				*S* 221	3·71	160–163	289·4	76·9
					S 222	5·45	163–164	291·8	83·1
					S 223	7·42	Residue	302·1	110·5
(ii) Esters of "liquid" acids *L*.									
					L 11	2·55	110–134°	251·9	66·6
L 1	26·06	108–140°	272·4	103·4	*L* 12	7·30	134–140	266·1	82·6
					L 13	2·67	140	272·9	97·2
					L 14	6·10	Residue	278·2	119·9
					L 21	5·19	140–149	280·0	122·7
L 2	31·23	140–155	296·1	166·3	*L* 22	12·86	149	284·3	138·8
					L 23	3·53	149–161	298·3	183·5
					L 24	5·47	Residue	310·1	238·1
					L 31	6·36	135–163	304·3	208·4
L 3	25·31	155–178	—	247·7	*L* 32	10·01	163–164	316·1	257·8
					L 33	4·65	Residue	329·0	269·9
					L 41	6·12	160–187	321·0	275·4
L 4	21·63	178–188	—	—	*L* 42	7·19	187	324·0	276·9
					L 43	4·54	Residue	337·0	272·3
L 5	9·23	Residue	446·5*	159·2					

* Saponification equivalent of residual esters, freed from unsaponifiable material, *L* 5: 325·8.

Table IV *b* (*continued*).

(iii) Esters of "liquid" acids *LR*.

Primary fractions					Refractionations				
No.	g.	B.P./1 mm.	Sap. eq.	I.V.	No.	g.	B.P./1 mm.	Sap. eq.	I.V.
LR 1	14·07	110–146°	—	96·7	*LR* 11	5·30	116–134°	255·8	66·6
					LR 12	3·32	134–144	271·0	93·9
					LR 13	4·82	Residue	288·7	132·2
LR 2	21·43	146–155	278·2	140·2	*LR* 21	5·70	135–148	273·4	110·3
					LR 22	6·13	148–154	287·1	127·6
					LR 23	5·49	Residue	301·5	186·7
LR 3	16·34	155–170	—	202·4	*LR* 31	5·91	148–161	295·7	155·3
					LR 32	3·67	161–170	308·5	202·8
					LR 33	5·29	Residue	321·2	246·9
LR 4	24·92	170–178	309·9	269·8	*LR* 41	3·60	140–162	309·9	226·3
					LR 42	8·22	162–164	317·5	258·4
					LR 43	8·70	Residue	332·8	275·6
LR 5	10·30	Residue	351·7*	262·5					

* Saponification equivalent of residual esters, freed from unsaponifiable material, *LR* 5: 328·4.

Table IV *c*. *Estimated composition of fatty acids.*

Acids	"Solid" acids *S* (46·5 %)	"Liquid" acids *L* (32·2 %)	"Liquid" acids *LR* (21·3 %)	Total %	Fatty acids	
					%	(mean unsaturation)
Saturated:						
Myristic	2·5	0·5	0·5	3·5	3·5	
Palmitic	9·9	0·3	Trace	10·2	10	
Unsaturated:						
C_{14} group	—	0·3	0·2	0·5	0·5	
C_{16} ,,	4·5	7·6	3·2	15·3	15·5	(−2·0 H)
C_{18} ,,	10·2	7·7	6·6	24·5	25	(−2·9 H)
C_{20} ,,	10·6	12·2	8·3	31·1	31·5	(−6·1 H)
C_{22} ,,	8·8	2·8	2·3	13·9	14	(−6·4 H)
Unsaponifiable	—	0·8	0·2	1·0	—	

The composition is not very different from that of the Newfoundland oil, although distinctly more of the unsaturated C_{20} and C_{22} acids are present, at the expense of unsaturated C_{16} and C_{18} acids. Palmitoleic acid is practically the only unsaturated C_{16} acid present, the C_{18} group consists of oleic and linoleic acids in nearly equal proportions, and, as in the previous analysis, the actual mean unsaturation of the C_{20} and C_{22} groups is higher than the figures indicate; gadoleic and cetoleic acids are both present, probably in definitely higher proportions than in the Newfoundland oil.

NORWEGIAN COD-LIVER OIL.

Table V *a*. *Lead salt separation.*

	g.	%	Sap. eq.	I.V.
"Solid" acids *S*	**168·8**	**29·6**	**278·5**	**95·7**
"Liquid" acids *L*	**401·8**	**70·4**	**298·5**	**170·2**

Table V *b*. *Fractionation of methyl esters.*

	Primary fractions				Refractionations				
No.	g.	B.P./1 mm.	Sap. eq.	I.V.	No.	g.	B.P./1 mm.	Sap. eq.	I.V.
				(i) Esters of "solid" acids *S*.					
S 1	44·59	70–135°	262·2	32·1	*S* 11	11·30	50–124°	249·1	23·9
					S 12	10·53	124–128	262·0	26·6
					S 13	8·38	128–135	268·3	30·4
					S 14	8·03	Residue	277·0	48·5
S 2	25·03	135–155	287·3	65·8	*S* 21	4·54	60–132	272·9	45·2
					S 22	7·04	132–135	283·1	56·9
					S 23	9·02	Residue	295·9	79·2
S 3	35·96	155–168	312·1	95·4	*S* 31	13·69	85–152	302·1	88·4
					S 32	8·17	152–168	312·6	93·0
					S 33	8·36	Residue	323·7	103·6
S 4	10·46	168–169	326·0	110·1					
S 5	21·50	Residue	326·5	148·9					
				(ii) Esters of "liquid" acids *L*.					
L 1	49·56	100–135°	269·8	74·5	*L* 11	13·89	70–133°	252·1	53·3
					L 12	12·70	135–141	262·7	73·9
					L 13	13·60	Residue	271·5	95·3
L 2	47·25	135–150	289·0	111·2	*L* 21	21·33	100–141	271·6	92·2
					L 22	23·82	141–151	280·5	105·3
					L 23	23·33	Residue	295·3	135·4
L 3	51·51	150–160	304·4	142·0	*L* 31	15·90	108–145	288·0	121·6
					L 32	13·67	145–155	294·9	131·6
					L 33	16·73	Residue	301·1	161·2
L 4	68·62	160–175	322·1	175·3	*L* 41	22·69	110–150	301·2	149·8
					L 42	24·48	150–160	314·0	168·0
					L 43	15·41	Residue	323·1	195·6
L 5	76·85	175–190	336·5	220·8	*L* 51	8·75	110–180	308·7	178·0
					L 52	34·22	180–190	327·5	201·4
					L 53	19·16	190	340·3	219·9
					L 54	9·20	Residue	349·9	235·5
L 6	26·97	Residue	375·6*	195·1					

* Saponification equivalent of residual esters, freed from unsaponifiable material, *L* 5: 336·5.

Table V *c*. *Determination of saturated components of ester fractions by oxidation.*

	Weight taken	Saturated esters present	
No.	g.	g.	Sap. eq.
L 11	8·10	3·71	242·0
L 21	12·80	2·50	273·1
L 22	18·93	1·94	274·2

Table V *d*. *Estimated composition of fatty acids.*

Acids	"Solid" acids *S* (29·6 %)	"Liquid" acids *L* (70·4 %)	Total %	Fatty acids %	(mean unsaturation)
Saturated:					
Myristic	2·7	2·1	4·8	5	
Palmitic	5·1	1·3	6·4	6·5	
Stearic	—	0·2	0·2	Trace	
Unsaturated:					
C_{14} group	—	0·4	0·4	0·5	
C_{16} ,,	3·1	13·1	16·2	16	(−2·0 H)
C_{18} ,,	7·4	23·2	30·6	31	(−2·8 H)
C_{20} ,,	10·5	19·7	30·2	30·5	(−5·1 H)
C_{22} ,,	0·8	9·7	10·5	10·5	(?)
Unsaponifiable	—	0·6	0·6		

The composition is similar to that of the other cod-liver oils studied, although the content of saturated acids is somewhat less. Palmitoleic acid is again practically the only unsaturated C_{16} acid present, the proportion of oleic to linoleic acid is much the same as in the Newfoundland oil examined; in the C_{20} and C_{22} groups of unsaturated acids, gadoleic acid occurs in some quantity, but the presence of cetoleic acid is doubtful, and polymerisation during heating prevented any reliable estimate being given for the average unsaturation of these groups of fatty acids.

Coalfish (Saith)-liver oil I.

Table VI *a*. *Lead salt separation.*

	g.	%	Sap. eq.	I.V.
"Solid" acids *S*	154·5	33·3	268·7	68·6
"Liquid" acids *L*	309·5	66·7	291·8	200·9

Table VI *b*. *Fractionation of methyl esters.*

Primary fractions					Refractionations				
No.	g.	B.P./1 mm.	Sap. eq.	I.V.	No.	g.	B.P./1 mm.	Sap. eq.	I.V.
(i) Esters of "solid" acids *S*.									
S 1	51·37	120–130°	259·0	14·1	*S* 11	3·27	105–123°	245·7	7·3
					S 12	14·64	123–125	251·1	7·1
					S 13	22·27	125–127	264·0	12·1
					S 14	6·02	Residue	274·3	36·9
S 2	32·38	130–140	275·0	27·7	*S* 21	14·57	130–131	265·9	16·0
					S 22	13·32	Residue	275·7	38·4
S 3	29·99	140–160	295·4	75·0	*S* 31	7·19	130–145	281·8	50·9
					S 32	10·46	145–147	287·9	66·8
					S 33	7·97	Residue	302·8	105·7
S 4	22·32	160–183	329·8	151·3	*S* 41	4·06	155–175	320·0	122·8
					S 42	4·40	175–177	320·2	141·3
					S 43	9·16	Residue	330·2	162·2
S 5	9·83	Residue	342·2*	188·4					
(ii) Esters of "liquid" acids *L*.									
L 1	60·39	130–150°	271·1	102·3	*L* 11	8·11	120–130°	255·1	58·8
					L 12	36·85	130–132	269·7	90·3
					L 13	7·58	Residue	290·5	141·2
L 2	89·58	150–160	292·7	148·5	*L* 21	14·21	135–141	270·4	107·3
					L 22	31·55	141–145	289·6	123·8
					L 23	25·00	145–146	296·2	139·1
					L 24	11·03	Residue	312·5	198·5
L 3	77·33	160–174	314·7	215·0	*L* 31	3·34	121–150	291·7	146·5
					L 32	34·55	150–151	298·9	173·6
					L 33	18·57	151–162	315·8	200·9
					L 34	15·09	Residue	327·9	229·8
L 4	54·64	174–197	335·9	230·8	*L* 41	3·91	150–173	313·4	222·8
					L 42	24·56	173–174	330·1	249·0
					L 43	11·26	174–178	335·2	256·4
					L 44	9·10	Residue	342·7	261·3
L 5	25·70	Residue	387·1*	217·2					

* Saponification equivalents of residual esters, freed from unsaponifiable material, *S* 5: 329·9, *L* 5: 337·1.

Table VI *c*. *Determination of saturated components of ester fractions by oxidation.*

No.	Weight taken g.	Saturated esters present g.	Sap. eq.
S 12	10·66	8·37	250·6
S 21	10·71	9·00	266·0
S 32	6·72	2·91	283·6
L 11	3·86	1·67	242·0
L 12	32·49	6·77	255·8
L 23	18·96	nil	—

Table VI *d*. *Estimated composition of fatty acids.*

Acids	"Solid" acids *S* (33·3 %)	"Liquid" acids *L* (66·7 %)	Total %	Fatty acids %
Saturated:				
Myristic	4·6	1·9	6·5	6·5
Palmitic	12·0	0·9	12·9	13
Stearic	0·6	—	0·6	0·5
Unsaturated:				
C_{16} group	3·1	11·3	14·4	14·5
C_{18} ,,	5·2	25·1	30·3	31
C_{20} ,,	6·3	18·1	24·4	24·5
C_{22} ,,	1·4	8·7	10·1	10
Unsaponifiable	0·1	0·7	0·8	—

The highly unsaturated esters from this oil were extremely susceptible to polymerisation, and the iodine values fell continuously at every operation involving heat; the cause of this behaviour is uncertain, but in consequence it is not possible to give estimates for the average unsaturation of the various groups. The C_{18} acids appear to be made up of approximately equal proportions of oleic and linoleic acids, whilst palmitoleic acid forms the greater part of the C_{16} group. On the whole the fatty acids are very similar in type and proportion to those of the cod-liver oils (especially, perhaps, the Newfoundland oil).

COALFISH (SAITH)-LIVER OIL II.

Table VII *a*. *Lead salt separation.*

	g.	%	I.V. (of corresponding methyl esters)
"Solid" acids *S*	92·0	31·5	57·5
"Liquid" acids *L*	200·0	68·5	203·1

Table VII *b*. *Fractionation of methyl esters.*

(i) Esters of "solid" acids *S*.

Primary fractions

No.	g.	B.P./1 mm.	Sap. eq.	I.V.
S 1	57·31	100–133°	268·7	24·42
S 2	14·92	133–155	311·0	101·6
S 3	6·18	155–165	333·3	140·5
S 4	8·00	Residue	339·7	146·3

Table VII *b* (*continued*).

(ii) Esters of "liquid" acids *L*.

Primary fractions					Refractionations				
No.	g.	B.P./1 mm.	Sap. eq.	I.V.	No.	g.	B.P./1 mm.	Sap. eq.	I.V.
L 1	56·51	120–143°	282·1	116·6	*L* 11	11·27	100–123°	262·3	77·1
					L 12	31·22	123–130	282·4	115·5
					L 13	8·74	Residue	291·8	177·1
L 2	73·31	143–163	309·4	196·4	*L* 21	7·07	120–145	290·5	140·6
					L 22	47·84	145–157	301·6	184·9
					L 23	13·45	Residue	326·8	271·8
L 3	51·23	163–180	336·7	277·4	*L* 31	4·72	145–170	322·5	260·1
					L 32	31·11	170–175	334·0	280·0
					L 33	11·03	Residue	340·9	288·5
L 4	14·62	Residue	372·1*	234·3					

* Saponification equivalent of residual esters, freed from unsaponifiable material, *L* 4: 327·6.

Table VII *c*. *Determination of saturated components of ester fractions by oxidation.*

		Saturated esters present	
No.	Weight taken g.	g.	Sap. eq.
S 1	18·45	14·03	260·4
S 2	11·52	3·50	288·3 (I.V. still 13·7)

Table VII *d*. *Estimated composition of fatty acids.*

Acids	"Solid" acids *S* (31·5 %)	"Liquid" acids *L* (68·5 %)	Total %	Fatty acids %	(mean unsaturation)
Saturated:					
Myristic	5·0	0·8	5·8	6	
Palmitic	12·0	—	12·0	12	
Stearic	0·2	—	0·2	Trace	
Unsaturated:					
C_{14} group	—	0·1	0·1	Trace	
C_{16} ,,	—	9·2	9·2	9·5	(–2·0 H)
C_{18} ,,	4·5	24·8	29·3	29·5	(–3·2 H)
C_{20} ,,	7·0	19·5	26·5	26·5	(–5·5 H)
C_{22} ,,	2·8	13·5	16·3	16·5	(–7·3 H)
Unsaponifiable	—	0·6	0·6	—	

In contrast to the experience in the previous analysis (saith oil I), little or no tendency to polymerisation was found when the methyl esters of the highly unsaturated C_{20} and C_{22} acids were distilled; the cause of this difference in behaviour is not clear. The total composition of the fatty acids differs but little from that observed in the first-named case, except that there is less palmitoleic acid present than usual, and correspondingly more of the acids of the C_{22} group—a feature which is in keeping with the saponification equivalents of the respective oils.

HAKE-LIVER OIL I.

Table VIII *a*. *Lead salt separation.*

	g.	%	I.V. (of corresponding methyl esters)
"Solid" acids *S*	50·0	30·0	46·3
"Liquid" acids *L*	117·0	70·0	182·0

Table VIII *b*. *Fractionation of methyl esters.*

(i) Esters of "solid" acids *S*.

Primary fractions

No.	g.	B.P./1 mm.	Sap. eq.	I.V.
S 1	17·82	110–130°	260·1	8·5
S 2	14·35	130–145	277·1	23·1
S 3	9·56	145–168	316·7	84·0
S 4	8·91	Residue	337·3	118·4

(ii) Esters of "liquid" acids *L*.

	Primary fractions				Refractionations				
No.	g.	B.P./1 mm.	Sap. eq.	I.V.	No.	g.	B.P./1 mm.	Sap. eq.	I.V.
L 1	19·31	117–137°	267·7	89·0	*L* 11	6·94	100–120°	254·6	57·2
					L 12	8·23	Residue	270·5	116·1
L 2	39·66	137–155	290·9	150·4	*L* 21	7·77	110–134	273·9	112·3
					L 22	19·14	134–139	286·6	141·5
					L 23	8·71	Residue	312·5	203·0
L 3	40·84	155–175	318·4	224·2	*L* 31	4·13	120–163	305·0	185·5
					L 32	21·89	163–167	316·3	215·0
					L 33	10·31	Residue	334·6	231·7
L 4	14·79	Residue	372·1*	235·4					

* Saponification equivalent of residual esters, freed from unsaponifiable material, *L* 4: 336·8.

Table VIII *c*. *Determination of saturated components of ester fractions by oxidation.*

	Weight taken	Saturated esters present	
No.	g.	g.	Sap. eq.
S 1	14·35	12·79	257·3
S 2	10·88	7·88	268·3
S 3	6·31	1·60	268·3

Table VIII *d*. *Estimated composition of fatty acids.*

Acids	"Solid" acids *S* (30·0 %)	"Liquid" acids *L* (70·0 %)	Total %	Fatty acids %	(mean unsaturation)
Saturated:					
Myristic	4·3	2·2	6·5	7	
Palmitic	12·7	—	12·7	13	
Unsaturated:					
C_{14} group	—	0·3	0·3	Trace	
C_{16} ,,	1·0	16·2	17·2	17	(−2·0 H)
C_{18} ,,	1·7	16·3	18·0	18	(−3·5 H)
C_{20} ,,	5·4	24·9	30·3	31	(−4·9 H)
C_{22} ,,	4·9	9·2	14·1	14	(?)
Unsaponifiable	—	0·9	0·9	—	

HAKE-LIVER OIL II.

Table IX *a*. *Lead salt separation.*

	g.	%	Sap. eq. (of corresponding methyl esters)	I.V.
"Solid" acids *S*	65·0	38·9	292·1	70·6
"Liquid" acids *L*	102·0	61·1	308·9	167·5

Table IX *b*. *Fractionation of methyl esters.*

(i) Esters of "solid" acids *S*.

	Primary fractions			
No.	g.	B.P./1 mm.	Sap. eq.	I.V.
S 1	9·88	100–130°	259·1	12·6
S 2	10·93	130–140	270·1	22·2
S 3	10·60	140–150	284·4	45·6
S 4	9·39	150–165	311·8	96·1
S 5	8·57	165–170	334·0	122·9
S 6	7·76	Residue	334·1	111·3

(ii) Esters of "liquid" acids *L*.

Primary fractions					Refractionations				
No.	g.	B.P./1 mm.	Sap. eq.	I.V.	No.	g.	B.P./1 mm.	Sap. eq.	I.V.
L 1	18·94	114–142°	274·8	88·8	*L* 11	5·70	110–125°	262·7	60·5
					L 12	3·29	125–130	265·3	83·1
					L 13	5·28	Residue	285·5	124·9
L 2	20·51	142–155	290·7	143·2	*L* 21	6·06	120–135	281·8	119·9
					L 22	4·81	135–144	290·3	142·5
					L 23	5·18	Residue	294·8	168·6
L 3	22·19	155–165	305·2	184·5	*L* 31	5·94	130–152	303·2	164·9
					L 32	6·05	152–157	315·2	182·2
					L 33	5·87	Residue	324·8	202·0
L 4	15·50	165–175	333·2	208·5					
L 5	15·00	Residue	396·8*	201·7					

* Saponification equivalent of residual esters, freed from unsaponifiable material, *L* 5: 334·8.

Table IX *c*. *Determination of saturated components of ester fractions by oxidation.*

		Saturated esters present	
No.	Weight taken g.	g.	Sap. eq.
S 2	7·21	5·53	262·8
S 3	6·90	3·32	273·6

Table IX *d*. *Estimated composition of fatty acids.*

				Fatty acids	
Acids	"Solid" acids *S* (38·9 %)	"Liquid" acids *L* (61·1 %)	Total %	%	(mean unsaturation)
Saturated:					
Myristic	3·6	1·0	4·6	4·5	
Palmitic	10·8	1·2	12·0	12	
Stearic	0·7	—	0·7	0·5	
Unsaturated:					
C_{16} group	0·9	10·6	11·5	12	(−2·0 H)
C_{18} ,,	8·1	18·3	26·4	27	(−3·0 H)
C_{20} ,,	9·7	19·6	29·3	30	(−4·1 H)
C_{22} ,,	5·1	8·8	13·9	14	(−5·4 H)
Unsaponifiable	—	1·6	1·6	—	

HAKE-LIVER OIL III.

Table X *a*. *Lead salt separation.*

	g.	%	Sap. eq.	I.V. (of corresponding methyl esters)
"Solid" acids *S*	53·0	28·8	287·2	42·5
"Liquid" acids *L*	131·4	71·2	310·5	146·5

Table X *b*. *Fractionation of methyl esters.*

(i) Esters of "solid" acids *S*.

Primary fractions

No.	g.	B.P./1 mm.	Sap. eq.	I.V.
S 1	5·82	100–130°	255·5	9·7
S 2	8·57	130–133	264·2	11·6
S 3	11·82	133–155	278·5	22·1
S 4	12·75	155–175	310·9	66·4
S 5	4·95	Residue	338·5	88·3

(ii) Esters of "liquid" acids *L*.

Primary fractions					Refractionations				
No.	g.	B.P./1 mm.	Sap. eq.	I.V.	No.	g.	B.P./1 mm.	Sap. eq.	I.V.
L 1	23·36	100–150°	273·0	86·8	*L* 11	6·18	80–115°	255·0	45·9
					L 12	5·88	115–121	267·0	77·9
					L 13	7·42	Residue	292·7	126·7
L 2	17·54	150–160	291·0	128·0					
L 3	37·24	160–170	312·7	159·3	*L* 31	11·29	110–150	297·5	142·6
					L 32	12·04	150–160	303·8	158·2
					L 33	9·49	Residue	332·7	168·1
L 4	20·15	170–180	335·2	195·4	*L* 41	6·61	140–156	322·9	172·1
					L 42	9·15	Residue	340·0	184·1
L 5	16·30	Residue	385·0*	169·1					

* Saponification equivalent of residual esters, freed from unsaponifiable material, *L* 5: 341·6.

Table X *c*. *Determination of saturated components of ester fractions by oxidation.*

No.	Weight taken g.	Saturated esters present g.	Saturated esters present Sap. eq.
S 2	4·04	3·10	262·1
S 3	6·02	4·01	272·4
L 2	13·14	1·20	262·2

Table X *d*. *Estimated compositior of fatty acids.*

Acids	"Solid" acids *S* (28·8 %)	"Liquid" acids *L* (71·2 %)	Total	Fatty acids %
Saturated:				
Myristic	2·9	2·6	5·5	5·5
Palmitic	9·4	1·6	11·0	11
Stearic	0·5	—	0·5	0·5
Unsaturated:				
C_{16} group	1·7	6·3	8·0	8
C_{18} ,,	6·3	27·1	33·4	34
C_{20} ,,	6·0	18·4	24·4	25
C_{22} ,,	2·0	14·0	16·0	16
Unsaponifiable	—	1·2	1·2	—

The highly unsaturated esters of the C_{20} and C_{22} groups showed considerable tendency to polymerise during distillation in the case of all three hake-liver oils, and the mean unsaturation of the corresponding fatty acids is probably somewhat greater than that suggested in the analysis of hake-liver oil II. The other acids present were similar in type to those of the cod- and saith-liver oils, and the proportions of all the acids were for the most part of the same order as those in the last-mentioned liver oils.

LING-LIVER OIL.

Table XI *a*. *Lead salt separation.*

	g.	%	I.V. (of corresponding methyl esters)
"Solid" acids *S*	41·5	22·5	27·3
"Liquid" acids *L*	142·5	77·5	164·6

Table XI *b*. *Fractionation of methyl esters.*

(i) Esters of "solid" acids *S*.

	Primary fractions			
No.	g.	B.P./1 mm.	Sap. eq.	I.V.
S 1	24·21	107–123°	262·1	7·9
S 2	8·60	123–142	280·5	32·2
S 3	8·61	Residue	317·1	84·8

(ii) Esters of "liquid" acids *L*.

Primary fractions					Refractionations				
No.	g.	B.P./1 mm.	Sap. eq.	I.V.	No.	g.	B.P./1 mm.	Sap. eq.	I.V.
L 1	29·51	113–139°	266·2	90·6	*L* 11	5·29	100–124°	256·1	58·0
					L 12	10·27	124–125	268·1	82·5
					L 13	10·50	Residue	288·5	118·9
L 2	47·70	139–153	291·5	131·3	*L* 21	5·62	126–139	278·3	106·2
					L 22	31·05	139–140	292·5	124·8
					L 23	6·48	Residue	312·0	196·4
L 3	44·72	153–175	315·4	210·7	*L* 31	5·05	140–155	299·0	154·8
					L 32	22·12	155–160	315·9	198·2
					L 33	14·22	Residue	335·7	249·8
L 4	5·83	175–182	337·0	254·6					
L 5	11·64	Residue	403·4*						

* Saponification equivalent of residual esters, freed from unsaponifiable material, *L* 5: 335·3.

Table XI *c*. *Determination of saturated components of ester fractions by oxidation.*

	Weight taken	Saturated esters present	
No.	g.	g.	Sap. eq.
S 1	19·73	18·03	260·9
S 2	5·05	3·46	271·9
S 3	3·01	0·70	283·2

Table XI *d*. *Estimated composition of fatty acids.*

Acids	"Solid" acids *S* (22·5 %)	"Liquid" acids *L* (77·5 %)	Total %	Fatty acids %	(mean unsaturation)
Saturated:					
Myristic	3·6	1·2	4·8	5	
Palmitic	11·9	0·9	12·8	13	
Stearic	0·8	—	0·8	1	
Unsaturated:					
C$_{14}$ group	—	0·2	0·2	Trace	
C$_{16}$,,	0·8	11·8	12·6	13	(– 2·0 H)
C$_{18}$,,	1·5	30·9	32·4	32·5	(– 2·8 H)
C$_{20}$,,	3·1	20·6	23·7	24	(– 5·7 H)
C$_{22}$,,	0·8	10·8	11·6	11·5	(– 7·0 H)
Unsaponifiable	—	1·1	1·1	—	

The ling-liver fatty acids, like those from hake, bear a general similarity to those of cod-liver oil. Palmitoleic is substantially the only unsaturated C_{16} acid present, whilst the C_{18} acids are a mixture of about 60 % oleic and 40 % linoleic acid; little polymerisation, as evidenced by falling iodine values, took place during distillation of the ling unsaturated esters and the figures given for the average unsaturation of the C_{20} and C_{22} groups are probably fairly accurate in this instance. Some monoethylenic C_{20} acid was present, but only very small quantities of the corresponding C_{22} acid.

SKATE-LIVER OIL.

Table XII *a*. *Lead salt separation.*

	g.	%	I.V. (of corresponding methyl esters)
"Solid" acids *S*	38·5	21·9	20·9
"Liquid" acids *L*	137·0	78·1	249·4

Table XII *b*. *Fractionation of methyl esters.*

(i) Esters of "solid" acids *S*.

	Primary fractions			
No.	g.	B.P./1 mm.	Sap. eq.	I.V.
S 1	30·47	110–133°	268·1	7·5
S 2	6·55	Residue	321·2	80·6

(Fraction *S* 1 (25·72 g.) gave 24·79 g. of saturated esters (sap. eq. 263·8) on oxidation.)

(ii) Esters of "liquid" acids *L*.

Primary fractions					Refractionations				
No.	g.	B.P./1 mm.	Sap. eq.	I.V.	No.	g.	B.P./1 mm.	Sap. eq.	I.V.
L 1	46·98	130–160°	283·9	not done	*L* 11	4·71	103–124°	265·5	83·0
					L 12	24·11	124–134	280·5	120·6
					L 13	8·68	134–136	303·3	186·7
					L 14	5·87	Residue	323·2	265·9
L 2	46·30	160–190	329·0	,,	*L* 21	7·65	130–155	296·6	203·0
					L 22	29·29	155–162	312·6	272·7
					L 23	6·43	Residue	336·0	308·0
L 3	31·11	190–196	333·4	,,	*L* 31	5·14	145–175	325·7	307·0
					L 32	14·05	175–178	335·4	335·4
					L 33	7·65	Residue	341·7	339·9
L 4	10·60	Residue	347·8*	,,					

* Saponification equivalent of residual esters, freed from unsaponifiable material, *L* 4: 331·6.

Table XII *c*. *Estimated composition of fatty acids.*

Acids	"Solid" acids *S* (21·9 %)	"Liquid" acids *L* (78·1 %)	Total %	Fatty acids %	(mean unsaturation)
Saturated:					
Myristic	3·5	0·2	3·7	4	
Palmitic	13·8	—	13·8	14	
Unsaturated:					
C_{14} group	—	Trace	Trace	Trace	
C_{16} ,,	—	10·5	10·5	10·5	(–2·0 H)
C_{18} ,,	1·0	19·7	20·7	20·5	(–3·3 H)
C_{20} ,,	3·6	29·0	32·6	32·5	(–7·3 H)
C_{22} ,,	—	18·4	18·4	18·5	(–9·5 H)
Unsaponifiable	—	0·3	0·3	—	

The skate-liver fatty acids stand out in the present series by reason of the high average unsaturation of the C_{22} and C_{20} groups, and equally by the absence of polymerisation during distillation of the corresponding esters. The oil also contains somewhat more of the C_{22} acids than the previous cases, and cetoleic acid is apparently absent; gadoleic acid is present, the C_{18} unsaturated acids are made up of about one-third oleic and two-thirds linoleic acid, and palmitoleic acid is substantially the only C_{16} unsaturated acid present.

DOGFISH-LIVER OIL.

Table XIII *a*. *Lead salt separation.*

	g.	%	Sap. eq.	I.V.
"Solid" acids *S*	110·0	33·2	286·2	56·0
"Liquid" acids *L*	221·0	66·8	361·0	139·0

Table XIII *b*. *Fractionation of methyl esters.*

Primary fractions					Refractionations				
No.	g.	B.P./1 mm.	Sap. eq.	I.V.	No.	g.	B.P./1 mm.	Sap. eq.	I.V.
(i) Esters of "solid" acids *S*.									
S 1	13·46	115–134°	252·5	11·3					
S 2	21·27	134–144	270·2	16·1	*S* 21	8·04	115–134°	260·7	8·6
					S 22	4·18	134	262·7	11·6
					S 23	6·38	Residue	274·2	26·7
S 3	13·65	144–154	284·1	32·3					
S 4	8·88	154–165	299·8	54·5					
S 5	26·25	165–184	334·1	72·1	*S* 51	5·92	154–174	318·7	67·3
					S 52	7·51	174–175	326·4	71·1
					S 53	2·21	175	337·3	72·7
					S 54	6·65	Residue	340·5	71·1
S 6	3·96	184–187	344·2	74·6					
S 7	9·01	Residue	332·3	84·4					
(ii) Esters of "liquid" acids *L*.									
L 1	26·15	126–154°	276·0	84·1	*L* 11	7·09	108–130°	256·9	49·3
					L 12	6·40	130–138	267·8	68·7
					L 13	3·11	138–140	279·4	81·7
					L 14	5·91	Residue	296·0	101·3
L 2	32·60	154–165	300·0	122·1	*L* 21	13·94	120–145	285·0	99·7
					L 22	6·56	145–155	298·1	117·0
					L 23	2·05	155	305·0	123·9
					L 24	6·53	Residue	313·4	136·6
L 3	57·22	165–187	351·4*	153·0	*L* 31†	6·52	120–155	288·2	121·5
					L 32	11·20	155–162	304·1	130·0
					L 33	9·18	165–175	315·3	132·6
					L 34	10·50	Residue	338·3	147·4
L 4	66·82	Residue	532·5*	138·4	*L* 41†	3·44	80–145	312·7	93·0
					L 42	10·33	145–170	324·5	98·2
					L 43	16·66	Residue	426·0*	141·2

* Saponification equivalents of these fractions, freed from unsaponifiable material, *L* 3: 319·4; *L* 4: 381·6; *L* 43 367·5.

† Unsaponifiable constituents removed as far as possible by ether-extraction of hydrolysed fractions *L* 3 and *L* 4, recovered fatty acids reconverted to methyl esters which were refractionated (*L* 31–34 and *L* 41–43).

Table XIII *c*. *Estimated composition of fatty acids.*

Acids	"Solid" acids *S* (33·2 %)	"Liquid" acids *L* (66·8 %)	Total %	Fatty acids	
				%	(mean unsaturation)
Saturated:					
Myristic	4·0	1·2	5·2	6	
Palmitic	8·2	1·2	9·4	10·5	
Stearic	2·5	—	2·5	3	
Arachidic	0·2	—	0·2	Trace	
Unsaturated					
C_{16} group	1·3	6·9	8·2	9	(– 2·0 H)
C_{18} ,,	3·4	18·5	21·9	24·5	(– 2·3 H)
C_{20} ,,	9·2	16·5	25·7	29	(– 3·3 H)
C_{22} ,,	4·4	6·7	11·1	12	(– 4·0 H)
C_{24} ,,	—	5·3	5·3	6	(– 2·0 H)
Unsaponifiable	—	10·5	10·5	—	

In some ways the fatty acid composition of this oil is the most interesting of the whole series. The oil contained much more unsaponifiable matter than the rest, and squalene formed a considerable proportion of the non-fatty matter, but the oil was not by any means so rich in this hydrocarbon as many shark- and ray-liver oils which have been examined by other workers. Nevertheless the character of the fatty acids is different from those of the oils previously dealt with in this paper, and in many ways (as appears from the general summary on p. 288) the acids of this dogfish-liver oil are intermediate in type between those of the Gadidae and of the skate-liver oil on the one hand, and those of Elasmobranch oils of high squalene content on the other. Palmitoleic acid is less prominent than usual, the average unsaturation of the C_{20} and C_{22} acids is much lower than usual, and especially noteworthy is the appearance of a small amount of monoethylenic acid, $C_{24}H_{46}O_2$.

SUMMARY.

Comparison of the fatty acid compositions of various fish oils.

It may be said at once that no apparent correlation between the vitamin potencies of the oils studied and the composition of their fatty acids is revealed as a result of the foregoing analyses; the most that can be stated is that, in the cases examined, the highest vitamin potency was observed in liver oils from the family Gadidae, but this may be a more or less fortuitous observation.

It is interesting, however, to consider the analyses which have now been made, in conjunction with a few other results of a similar nature which are already available, when classified from a biological standpoint. This has been done in Table XIV: the data for the whale oils (*Balaena* sp.), menhaden oil and Japanese sardine oil are taken from a communication by Armstrong and Allan [1924], those for the liver oils of *Scymnorhinus* and allied genera were given by Hilditch and Houlbrooke [1928], those for the head and blubber oils of the sperm whale by Hilditch and Lovern [1928], and for sperm-liver oil by Tsujimoto and Kimura [1928].

The figures in Table XIV are perhaps chiefly valuable at the moment in suggesting the desirability of adding as rapidly as possible to the meagre number of quantitative data of the kind which have so far been collected. Investigations of this kind are necessarily lengthy and tedious, but the results so far obtained indicate the possibility that they may lead to useful correlations as well as differentiations between the build of the fats occurring in different classes of marine animals or, for example, present in different parts of the same animal.

Inspection of Table XIV will show that, leaving apart minor differences in the percentages of the fatty acids concerned, the liver oils of the Gadidae and the flesh oils of the two members of the herring family stand apart from the rest in the approximate constancy of their general composition, the range of which is as follows:

	%	Mean unsaturation
Myristic acid	3·5–7	—
Palmitic acid	6·5–13	—
Stearic acid	nil–1	—
Myristoleic acid	Trace–0·5	(– 2·0 H)
Palmitoleic acid	13–20	(– 2·0 to – 3·0 H)
Oleic and linoleic acids	18–32·5	(– 2·8 to – 4·0 H)
*Unsaturated C_{20} acids	19–31·5	(– 4·9 to – 7·3 H)
*Unsaturated C_{22} acids	10–19	(– 5·4 to – 10·0 H)

The blubber fats of the ordinary whales approach most closely in composition to the foregoing, but on the whole appear to contain greater amounts of oleic and linoleic acids, with corresponding reduction in the total proportions of polyethylenic C_{20} and C_{22} acids, the total percentage of saturated acids being little different in either group. The interesting, but only partially quantitative, study by Tsujimoto and Kimura [1928] of the fatty acids of the liver of the sperm whale suggests that here also the composition is much the same as in the body fats of the Balaenidae and Clupeidae and the liver fats of the Gadidae, and these authors also state that the fat as obtained contains only 16 % of unsaponifiable matter, of which 8 % is cholesterol, higher fatty alcohols being probably absent; sperm head- and blubber-oils, on the contrary, contain 33–40 % of combined higher fatty alcohols, whilst the fatty acids also present are entirely different in character from those of any other marine animal [Hilditch and Lovern, 1928].

Whilst differences of the latter kind point clearly to synthesis of particular varieties of fatty compounds by specific classes of animals, the general similarity in composition, previously referred to, of the fatty acids of the livers and

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* The relative amounts of monoethylenic acids $C_{20}H_{38}O_2$ and $C_{22}H_{42}O_2$ appear to vary somewhat widely from one species to another, and it is not certain that all the highly unsaturated acids of these groups are the same, even in liver oils of the same species of fish. At all events, it was noticeable that in certain cases (for example, Norwegian cod, saith (1), and hake) the esters of these acids polymerised exceedingly readily, whilst in others (especially saith (2), ling and skate) there was practically no tendency to polymerisation and fall in iodine value. This may be due (cf. p. 270) to some accidental catalytic acceleration of the polymerisation process from an unrecognised source, or may be an indication of structural differences in the constitution of the polyethylenic acids.

Table XIV. *Percentage composition of fatty acids of marine animal oils.*

Oil	Species	Saturated acids			Unsaturated acids				
		C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}	C_{24}
Sub-division TELEOSTOMI. Order TELEOSTEI.										
Family GADIDAE.										
Cod-liver (Newfoundland)	*Gadus morrhua*	6	8·5	0·5	Trace	20	29	26	10	—
Cod-liver (Scottish)	,, ,,	3·5	10	—	0·5	15·5	25	31·5	14	—
Cod-liver (Norwegian)	,, ,,	5	6·5	Trace	0·5	16	31	30·5	10·5	—
Coalfish (saith)-liver I	*G. virens*	6·5	13	0·5	—	14·5	31	24·5	10	—
,, ,, II	,,	6	12	Trace	Trace	9·5	29·5	26·5	16·5	—
Hake-liver I	*Merluccius merluccius*	7	13	—	Trace	17	18	31	14	—
,, II	,,	4·5	12	0·5	—	12	27	30	14	—
,, III	,,	5·5	11	0·5	—	8	34	25	16	—
Ling-liver	*Molva molva*	5	13	1	Trace	13	32·5	24	11·5	—
Family CLUPEIDAE.										
Japanese sardine (whole fish)	*Clupanodon melanostica*	6	10	2	—	13	24	26	19	—
Menhaden (whole fish)	*Alosa menhaden*	6	16	1·5	—	15·5	30	19	12	—
Sub-division ELASMOBRANCHII. Order SELACHII.										
Family SQUALIDAE.										
Dogfish-liver	*Squalus acanthias*	6	10·5	3	—	9	24·5	29	12	6*
Mixed liver oils from	*Centrophorus, Lepidorhinus,* etc. sp.	1	13	2·5	Trace	3·5	35·5	16·5*	16*	12*
Family DALATIIDAE.										
Liver oil of	*Scymnorhinus lichia*	1	14·5	3·5	0·5	4	29	10·5*	26*	10*
Family RAJIDAE.										
Skate-liver	*Raia maculata*	4	14	—	Trace	10·5	20·5	32·5†	18·5†	—
Division MAMMALIA. Order CETACEA.										
Family BALAENIDAE.										
Whale oil (blubber):										
Arctic	*Balaena mysticetus*	4	10·5	3·5	—	18	33	20	11	—
Newfoundland	,,	7·5	10	3	1·5	18	44	—	16	—
South Sea	*Balaena australis*	8	12	2	1·5	15	43	8	10·5	—
Family PHYSETERIDAE.										
Sperm whale:										
Head‡	*Physeter macrocephalus*	14	8	2	14	15	17*	6·5*	—	—
Blubber	,,	5	6·5	—	4	26·5	37*	19*	—	—
Liver	,,	(24·6 % saturated)			(42 % monoethylenic and 23·4 % polyethylenic)					

* Practically only monoethylenic acids present.

† Particularly high average unsaturation.

‡ The head acids also contained 3·5 % capric, 16 % lauric and 4 % lauroleic acids; the blubber acids 1–2 % of lauric acid.

in some cases the body-oils of many marine animals of diverse biological orders suggests that fish and marine mammalia may derive a considerable proportion of their fats by direct assimilation. The polyethylenic acids of the C_{20} and C_{22} series, which at one time were known only as characteristic of fishes and marine mammals, have been detected by Tsujimoto [1925] in Algae of different kinds in every instance tested, although it is true that the proportion present is much lower than in the fish-liver oils which are the subject of this paper; furthermore, it is stated by Grün and Halden [1929] that the fat of birds which feed on fish also contains these highly-unsaturated acids, which are not present in the fat of birds whose diet is confined to insects, grain, etc. from non-marine sources. The determination of the origin (evidently marine in nature) of these unique highly unsaturated fatty acids is thus a problem of great interest which remains to be solved.

Finally, the detailed composition of the fatty acids of the Elasmobranchii deserves further attention, in view of the interesting results so far obtained.

It will be noticed that, in this group, the acids of skate-liver oil alone resemble those of the more frequently occurring type (but possess distinctly greater average unsaturation and amount of polyethylenic C_{20} and C_{22} acids, whilst the monoethylenic acids of these series are only present in minimal quantities); this skate-liver oil, however, contains, for an Elasmobranch oil, a very low proportion of unsaponifiable matter—lower, indeed, than any of the Gadidae oils.

At the other extreme are the fats of *Centrophorus*, *Lepidorhinus* and *Scymnorhinus* species, which were the complement of large proportions of unsaponifiable matter composed mainly of the hydrocarbon squalene; the fatty acids from these sources contained no highly unsaturated C_{20} and C_{22} derivatives, but were mainly ethylenic in character and notable amounts of the monoethylenic acid containing 24 carbon atoms (selacholeic acid [Tsujimoto 1927]) were also present.

The dogfish-liver oil reported upon in this paper was intermediate in character between these varieties of Elasmobranch oil: it contained 10·5 % of unsaponifiable matter, which consisted partly of liquid hydrocarbons (squalene) and partly of solid crystalline compounds (cholesterol?). The fatty acids present were also intermediate in composition between those of the two extreme types, for selacholeic acid was present in small quantity, whilst the mean unsaturation of the C_{20} and C_{22} acids (about − 4·0 H) was higher than monoethylenic but less marked than in the cod- and other fish-liver oils which were first discussed.

So far as the present data go, therefore, there is a definite and close relation in the Elasmobranch group between the character of the fat present and the amount and nature of the unsaponifiable matter. We are not inclined at this juncture to do more than point out that our recent results strengthen the conclusion of Hilditch and Houlbrooke [1928] that there is some connection between the absence of the polyethylenic C_{20} and C_{22} acids so characteristic of most animal oils and the presence of large proportions of squalene. We believe that at present no suggestion can be usefully offered to explain the genesis, much less a hypothetical transmutation into one another, of either the polyethylenic acids, of squalene, or of cholesterol or other sterols which are frequent components of the non-fatty matter. Bearing in mind what is already known of the fundamental differences in chemical structure of these compounds (the normal aliphatic carbon chain present in the polyethenoid C_{20} and C_{22} acids; the aliphatic branched chain system of squalene, $C_{30}H_{50}$, built up from isoprene units; and the complex condensed ring-systems present in cholesterol), it is very difficult to imagine that they are mutually interconvertible by ordinary biochemical processes. It is perhaps more opportune, for the moment, to speculate whether different metabolic processes characteristic of specific organisms may not lead, according to circumstances, to the accumulation of differing kinds of organic compounds and the disappearance of other forms which are further utilisable by the organism concerned.

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XXXV. THE EFFECT OF GLUTATHIONE ON THE DETERMINATION OF BLOOD-SUGAR.

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In an earlier paper [Herbert and Groen, 1929] it was shown that the discrepancies between the results of blood-sugar methods were due to non-glucose reducing substances present mainly in corpuscles. It appeared that ferric hydroxide filtrates and zinc hydroxide filtrates contained little or none of the non-glucose material, and that therefore the original MacLean method and the original Hagedorn-Jensen method gave figures close to the true sugar value. The non-sugar material was shown to be present in tungstic acid filtrates, and to have a marked effect on all the analytical methods applied to tungstic acid filtrates except that of Benedict [1928]; the Hagedorn-Jensen method, applied to tungstic acid filtrates, was affected most of all. It was suggested that the principal non-glucose substance responsible was glutathione.

In the present investigation the effect of pure reduced glutathione on blood-sugar methods has been studied. The work falls into two sections; (1) a study of the effect of glutathione on the analytical methods, when no treatment corresponding to protein precipitation is used, and (2) a study of the behaviour of glutathione in various methods of protein precipitation, using mixtures of plasma and glutathione.

Two different samples of glutathione were employed, both of which were received from Sir Frederick Hopkins's laboratories. The first, received in December 1928, was a deliquescent substance, the solutions of which had a smell resembling that of mint. The second sample was received in July 1929, and consisted of the pure tripeptide of glycine, glutamic acid, and cysteine, prepared by Hopkins's new method [1929].

The following blood-sugar methods have been used.

(1) The Folin-Wu method [1920].
(2) The Shaffer-Hartmann method as modified by Somogyi [1926].
(3) The Hagedorn-Jensen method [1923].
(4) The Benedict method [1928].

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THE EFFECT OF PURE REDUCED GLUTATHIONE ON THE FERRICYANIDE REAGENT AND THE VARIOUS COPPER REAGENTS.

In this section of the work no treatment corresponding to protein precipitation was used; aqueous solutions containing glucose and glutathione were analysed by the various methods. For each method the quantities of glucose and glutathione taken were those which might be expected to be present when blood is analysed, assuming for the moment that all the blood-glutathione reaches the filtrate, and remembering that the amount of filtrate taken corresponds to 0·5 cc. blood in the Shaffer-Hartmann method, 0·2 cc. blood in the Folin-Wu and Benedict methods, and 0·1 cc. in the Hagedorn-Jensen method. In these analyses the amount of glucose was that corresponding to a blood-sugar of 100 mg. per 100 cc., and the amounts of glutathione those corresponding to 36 to 150 mg. glutathione per 100 cc. blood. In this way, the effect of the glutathione was determined as an increment above the true sugar, and the conditions were comparable with those obtaining in blood analyses, except for the omission of the protein-precipitation methods. The volume of solution was in each method the same as in blood analyses. Results are given in Tables I and II. Fig. 1 gives the results in Table II in graphic form. We have tabulated only our results with the pure tripeptide, in the presence of an amount of glucose corresponding to a blood-sugar figure of 100 mg. per 100 cc. The earlier sample of glutathione gave similar figures.

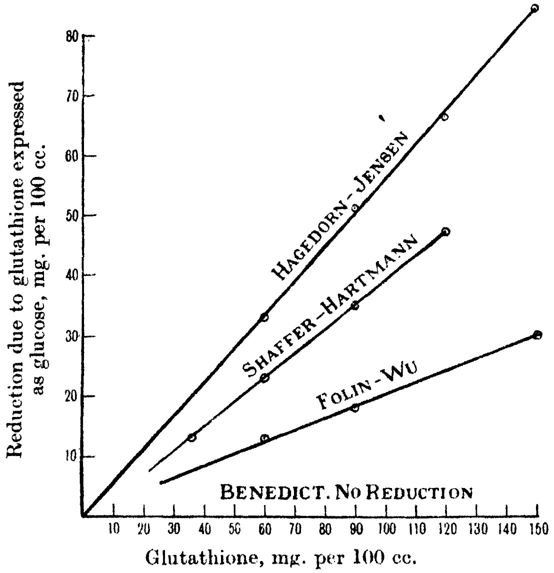

Fig. 1.

In other experiments the amount of glucose varied from 50 to 100 mg. per 100 cc., and the glucose equivalent of glutathione was the same. In the complete absence of glucose, however, the reduction due to glutathione was not in all methods the same as in the presence of glucose; this will be discussed separately for each method.

No special precautions were taken to prevent oxidation of the solutions by air, but in spite of this the results were, upon the whole, constant.

Table I. *Reducing action of glutathione in the presence of glucose. No treatment corresponding to protein precipitation.*

Method	Glutathione taken, mg.	Reduction due to glutathione cc. $N/200$ thiosulphate	 Glucose mg.
Hagedorn-Jensen	0·06	0·190	0·033
	0·09	0·290	0·051
	0·12	0·375	0·066
	0·15	0·475	0·084
Shaffer-Hartmann	0·18	0·55	0·065
	0·30	1·05	0·115
	0·45	1·55	0·175
	0·60	2·10	0·235
Folin-Wu	0·12	—	0·026
	0·18	—	0·036
	0·30	—	0·060
	0·48	—	0·090

Table II. *Glucose equivalents of various concentrations of glutathione (determined in the presence of glucose).*

	Glucose equivalent of glutathione, mg. per 100 cc.			
Glutathione, mg. per 100 cc.	Hagedorn-Jensen	Shaffer-Hartmann	Folin-Wu	Benedict [1928]
36	—	13	—	—
60	33	23	13	0
90	51	35	18	0
120	66	47	—	0
150	84	—	30	0
240	—	—	45	—

The Hagedorn-Jensen method.

In the presence of glucose, the ratio of the amount of glutathione present to its glucose equivalent is 100 : 56, and this ratio is constant for the range covered by the analyses. (Tables I and II.)

In the absence of glucose, the glucose equivalent of glutathione is a little higher, viz.:

$$0{\cdot}050 \text{ mg. G. SH} \equiv 0{\cdot}039 \text{ mg. glucose},$$
$$0{\cdot}100 \text{ mg. G. SH} \equiv 0{\cdot}070 \text{ mg. glucose}.$$

The relation of glutathione to thiosulphate (determined in the presence of glucose) is 0·1 mg. G. SH ≡ 0·32 cc. $N/200$ thiosulphate. This is greater than the theoretical quantity required solely for the oxidation of the sulphydryl group. On the basis of the tripeptide formula, in the oxidation of the —SH to the —SS— form, one molecule of glutathione (M.W. 307) corresponds to one atom of hydrogen, *i.e.* 0·1 mg. G. SH corresponds to 0·065 cc. $N/200$ thiosulphate. Therefore there must be some change other than the oxidation of glutathione from the —SH to the —SS— form; this would be expected when such an unstable compound is boiled in alkaline solution in the presence of an oxidising agent.

Glutathione has some reducing action on the Hagedorn-Jensen reagents at room temperature. If glutathione is added to the ferricyanide, and the iodide-zinc-sulphate-chloride solution and acetic acid are added at once, a variable reduction occurs, which is less than that observed when glutathione is heated with the ferricyanide, but greater than that theoretically required for the change from the —SH to the —SS— form. On the other hand, if the three reagents are mixed and the glutathione is added to the acid mixture, the reduction approaches the theoretical figure for the oxidation of the sulphydryl group.

Shaffer-Hartmann (Somogyi) method.

In the presence of glucose the ratio of glutathione to its glucose equivalent is 100 : 39, and this ratio is constant for the range covered by the analyses (Tables I and II).

In comparing the effects produced by glutathione in the presence and in the absence of glucose, it is necessary to consider a peculiarity in Somogyi's table representing the relation of glucose to thiosulphate for this method [Somogyi, 1926]. In this table, whereas throughout the major part of the range the relation of glucose to thiosulphate is constant (1 cc. *N*/200 thiosulphate is equivalent to 0·110 mg. glucose), in the lowest region of the table this relation does not hold, and 1 cc. *N*/200 thiosulphate is equivalent to 0·205 mg. glucose. When the reducing action of glutathione is determined in the presence of glucose, the glucose value corresponding to the glutathione is read as an increment in the upper part of the table; when the glutathione is analysed alone the lowest part of the table is used. It is found that the reduction attributed to glutathione, when expressed as glucose, remains almost the same in the absence as in the presence of glucose, although the thiosulphate corresponding to the glutathione is not the same, viz.:

in the presence of glucose

0·5 mg. G. SH ≡ 1·75 cc. *N*/200 ≡ 0·195 mg. glucose;

in the absence of glucose

0·5 mg. G. SH ≡ 0·85 cc. *N*/200 ≡ 0·185 mg. glucose.

For our purpose it is most convenient to assume, as we have done in calculating these figures, that in the mixture the reduction value of glucose remains the same, and the reduction value of glutathione is increased; but the actual mechanism of the reduction is unknown. When, in the Shaffer-Hartmann method, all the reduction values are expressed as glucose, the effect is obscured.

The relation of glutathione to thiosulphate (determined in the presence of glucose) is very similar in the Shaffer-Hartmann and Hagedorn-Jensen methods; viz. in the Shaffer-Hartmann method, 0·60 mg.G. SH is equivalent to 2·10 cc.

$N/200$ thiosulphate, and in the Hagedorn-Jensen method, 0·06 mg. G. SH is equivalent to 0·19 cc. $N/200$ thiosulphate.

Glutathione reduces the reagent at room temperature. To determine this reduction the glutathione and the copper reagent were mixed at room temperature, acidified at once and allowed to stand for 2 to 5 minutes; the mixture was then titrated in the usual way, when it was found that:

$$0{\cdot}50 \text{ mg. G. SH} \equiv 0{\cdot}36 \text{ cc. } N/200 \text{ thiosulphate,}$$
$$1{\cdot}00 \text{ mg. G. SH} \equiv 0{\cdot}76 \text{ cc. } N/200 \text{ thiosulphate.}$$

The requirement for the change from the —SH to the —SS— form is 1 mg. G. SH $\equiv$ 0·65 cc. $N/200$ thiosulphate. The oxidation of the sulphydryl group by iodine may therefore account for the reaction which occurs at room temperature, but there must be an additional reaction when the glutathione is heated with the copper reagent, as in the orthodox method of estimation.

The Folin-Wu method.

In the presence of glucose the ratio of glutathione to its glucose equivalent is 100 : 20, and this relation is constant for the range covered by the analyses (Tables I and II). The effect in the absence of glucose is the same (in this case the glucose standard is necessarily more dilute than for determinations in the presence of glucose). The presence of glutathione or of its oxidation products does not seem to cause any alteration in the blue colour, or to cause it to fade. The colours were matched as quickly as possible after dilution.

At room temperature glutathione reduces the copper reagent, as was previously noted by Herbert and Groen [1929]. This reduction in the cold is much less than that which occurs when glutathione is heated with the copper reagent.

The Benedict [1928] *method.*

In the presence of glucose, glutathione produces no measurable effect. In the absence of glucose, a very slight reduction of the reagent occurs (as indicated by the colour developed on addition of the phosphomolybdate), but with the amounts of glutathione handled, this was too small to be estimated.

The boiling-time was in all cases 10 minutes. There was a tendency for the colour to fade more rapidly in the glutathione mixtures than in the standard glucose solution, but no fading was observed within 10 minutes after dilution, during which time the readings were completed.

The results obtained with aqueous solutions show that the glucose equivalents of glutathione are in the approximate ratio 3 : 2 : 1 : 0 in the Hagedorn-Jensen, Shaffer-Hartmann, Folin-Wu, and Benedict methods respectively. Therefore when any blood filtrate which contains glutathione is analysed by these methods these effects will be observed. In considering actual blood analyses it is essential to know how the glutathione behaves at the stage of protein precipitation; this question will next be considered.

THE BEHAVIOUR OF PURE REDUCED GLUTATHIONE IN THE VARIOUS METHODS OF PROTEIN PRECIPITATION.

Mixtures of plasma and glutathione were submitted to the various processes for protein precipitation, and the filtrates were analysed by the different methods[1]. Since plasma contains no glutathione, the total quantity of glutathione in the mixture was known, and the results can legitimately be compared with those obtained with simple solutions (Table II and Fig. 1).

Tungstic acid filtrates. For all four methods the analysis of tungstic acid filtrates from mixtures of plasma and glutathione gave the same results as had been obtained on the corresponding aqueous solutions (glucose and glutathione). Table II and Fig. 1 may be applied to tungstic acid filtrates. We therefore conclude that the whole of the glutathione passes into the tungstic acid filtrates. As would be expected, the filtrates show a strongly positive nitroprusside reaction.

Zinc hydroxide filtrates. Two methods of preparing zinc hydroxide filtrates were employed: (1) the technique described by Hagedorn and Jensen for use with their ferricyanide method [1923], and (2) the technique of Somogyi [1929, 1, 2] which may be used in association with various analytical methods.

In the Hagedorn-Jensen precipitation method the blood is diluted 1 in 61 and is heated with the zinc hydroxide in a boiling water-bath before filtration; the relative quantities of zinc sulphate and sodium hydroxide are such that the filtrates are slightly acid (p_H 6·8). In Somogyi's precipitation technique the blood is laked in a solution of zinc sulphate and sulphuric acid, sodium hydroxide is added, and the mixture is filtered; the whole process is carried out at room temperature; the dilution of the blood is 1 in 10; and there is a slight excess of sodium hydroxide, so that the filtrate is slightly alkaline (p_H 7·4). Mixtures of plasma and glutathione have been submitted to both these processes, and the filtrates analysed by the ferricyanide method.

When the Hagedorn-Jensen precipitation technique was followed, the added glutathione caused a slight increment above the plasma-sugar figure, though this effect was much less than that observed with the tungstic acid precipitation. The figures obtained were:

60 mg. glutathione per 100 cc. ≡ 10 mg. glucose per 100 cc.
90 ,, ,, ,, 100 ,, ≡ 19 ,, ,, ,, 100 ,,
120 ,, ,, ,, 100 ,, ≡ 33 ,, ,, ,, 100 ,,

Evidently a small proportion of the added glutathione passed into these filtrates. With the larger quantities of glutathione, the filtrates showed a faintly positive nitroprusside reaction.

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[1] In using the ferricyanide method for filtrates other than the original zinc filtrates of Hagedorn and Jensen, we took an amount of filtrate corresponding to 0·1 cc. plasma, and diluted this to 12 cc. before heating with the alkaline ferricyanide. The conditions were therefore strictly comparable with those obtaining in the original method, in which the total amount of filtrate corresponds to 0·1 cc. of blood or plasma, and the volume of filtrate and washings is approximately 12 cc.

When Somogyi's precipitation method was used the added glutathione caused no increment above the plasma-sugar figure, and the filtrate showed no nitroprusside reaction. Therefore no glutathione passes into filtrates made by Somogyi's technique.

It was found that the difference in the behaviour of glutathione in the two methods of zinc hydroxide precipitation was due to the difference in reaction of the protein precipitation mixtures. If the amount of alkali in the Hagedorn-Jensen technique was increased (keeping other conditions the same) so that a slightly alkaline filtrate was obtained, no glutathione passed into the filtrate, as indicated by the figures given on analysis by the ferricyanide method. On the other hand, if the amount of alkali in Somogyi's technique was diminished, so as to produce a neutral filtrate, a part of the glutathione passed into the filtrate, as indicated by the effect on the ferricyanide reagent, and by the fact that a positive nitroprusside reaction was obtained.

From the above observations we can predict the effect which would be produced by a given amount of glutathione in blood when various analytical methods are applied to the various blood-filtrates. It is known that reduced glutathione is present in blood, and occurs only in the corpuscles [Hunter and Eagles, 1927; Holden, 1925; Thomson and Voegtlin, 1926; Uyei, 1926; Hopkins, 1929]. The exact quantity in human blood is not known; the figure given by Hunter and Eagles—40 to 50 mg. per 100 cc. whole blood—is based on the intensity of the nitroprusside reaction in tungstic acid filtrates, and may be taken as approximately correct if it may be assumed that glutathione is the only sulphydryl compound of which any appreciable amount is present in blood.

If there are 50 mg. reduced glutathione per 100 cc. of whole blood, and if this is the only non-glucose substance capable of producing an appreciable effect on blood-sugar methods, we should expect the following results when the various methods are applied to whole blood: on tungstic acid filtrates, the Hagedorn-Jensen figure would be higher than the true sugar value by 28 mg. per 100 cc.; the Shaffer-Hartmann figure would be too high by 19 mg. per 100 cc.; the Folin-Wu figure too high by 10 mg. per 100 cc. The Benedict method alone would give a true sugar figure for tungstic acid filtrates. All four analytical methods applied to the zinc hydroxide filtrates made by Somogyi's technique would give true sugar values. The original Hagedorn-Jensen method (*i.e.* the Hagedorn-Jensen precipitation technique together with the ferricyanide method of analysis) would give slightly high figures (about 9 mg. per 100 cc. above the true sugar value).

The comparison between these predicted effects and the results observed in blood analyses is given in the following paper.

SUMMARY.

The effect of aqueous solutions of reduced glutathione upon the various oxidising reagents employed in blood-sugar methods has been determined. The greatest effect (expressed in terms of glucose) is produced in the ferricyanide reduction; next in order come the Shaffer-Hartmann (Somogyi) copper reduction, and the Folin-Wu copper reduction. The copper reagent of Benedict is unaffected by glutathione.

In the tungstic acid precipitation, applied to mixtures of plasma and glutathione, all the added glutathione passes into the filtrates. In Somogyi's zinc hydroxide precipitation no glutathione passes into the filtrates. The zinc hydroxide precipitation of Hagedorn and Jensen allows a portion of the added glutathione to pass into the filtrates.

The blood-sugar determinations will be unaffected by glutathione (1) when any analytical method is applied to a filtrate containing no glutathione (*e.g.* Somogyi's zinc filtrate), or (2) when the analytical method employed is unaffected by glutathione, whatever the protein precipitation method (*e.g.* Benedict's method applied to any blood filtrate).

We wish to express our thanks to Sir Frederick Hopkins and to Mr Meldrum for the samples of glutathione.

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XXXVI. THE NON-SUGAR REDUCING SUBSTANCES OF HUMAN BLOOD, WITH SPECIAL REFERENCE TO GLUTATHIONE.

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In the preceding paper the behaviour of pure reduced glutathione in the various blood-sugar methods was described. In the present paper the effects produced by the non-sugar reducing substances of blood are determined, and are compared with those obtained with pure glutathione.

EXPERIMENTAL.

Freshly drawn, oxalated, venous blood of normal adults was used, and to each sample three methods of protein precipitation were applied. These were: (1) the Folin-Wu tungstic acid precipitation, which allows glutathione to pass into the filtrate, (2) the zinc hydroxide precipitation of Somogyi [1929, 1] in which the glutathione is removed, and (3) a new technique for preparing a glutathione-free filtrate with tungstic acid. Each of the three filtrates was analysed by the following methods: Hagedorn-Jensen [1923], Shaffer-Hartmann [Somogyi, 1926], Folin-Wu [1920] and Benedict [1928]. In all precipitation methods the dilution of the blood was 1 in 10, so that for the Shaffer-Hartmann, Folin-Wu, and Benedict methods the volumes of filtrate described in the original methods were taken. In the ferricyanide method 1 cc. filtrate was used, and the volume was made up to about 12 cc. with distilled water before addition of the ferricyanide. This procedure is exactly comparable with the original Hagedorn-Jensen method, in which 0·1 cc. of blood is taken, and the volume of the filtrate from this, together with the washings, is about 12 cc.

All filtrates were neutralised (to phenolphthalein) before use; the volume of alkali added was negligible in relation to the total volume. In the case of the zinc hydroxide filtrates the addition of the alkali caused a further precipitation of zinc hydroxide, which was removed by re-filtering.

In the colorimetric methods readings were taken within 10 to 15 minutes after dilution of the coloured solution, and no fading of the colour took place

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[1] Holding a grant from the Medical Research Council.

in this time. Everett [1929] has suggested that such fading may cause an error in the Benedict method.

Folin-Wu filtrates were made in the usual way [Folin and Wu, 1919]. They invariably gave a positive nitroprusside reaction.

Zinc hydroxide filtrates were made as described by Somogyi [1929, 1]. In every case we found the nitroprusside reaction negative. Therefore these filtrates contain no appreciable amount of reduced glutathione.

On the basis of our experience with glutathione solutions we may say that a negative nitroprusside reaction indicates that the concentration of glutathione, if any, is less than 0·3 mg. per 100 cc. filtrate. Such small amounts produce no measurable effect on the analytical methods.

A preliminary comparison was made between the Somogyi zinc precipitation method and the Hagedorn-Jensen zinc precipitation method, using the ferricyanide method of analysis. In every case the Hagedorn-Jensen precipitation technique gave a higher figure than the Somogyi precipitation technique. The difference was 1 to 10 mg. per 100 cc. (average 4 mg. per 100 cc.) and was therefore scarcely outside the limits of experimental error, but since the Hagedorn-Jensen filtrate gave always the higher figures, the difference is probably significant. On the basis of the work with pure glutathione, it was predicted that the Hagedorn-Jensen filtrate would give figures higher than the Somogyi filtrate by 9 mg. per 100 cc. The difference between the observed and expected results is small.

The zinc hydroxide filtrates in the experiments in Table I were all made by the Somogyi technique.

Modified tungstic acid filtrates. Glutathione is present only in the corpuscles, and therefore presumably exists in a non-diffusible form. It is possible to prepare a filtrate free from glutathione if the corpuscles are preserved intact during the precipitation process. The Folin-Wu tungstic acid procedure has been modified by delivering the blood sample into an isotonic salt solution instead of laking it in distilled water, and adding a quantity of tungstate and acid sufficient to precipitate the plasma proteins. The procedure is as follows.

1 cc. blood is mixed with 8 cc. 3 % sodium sulphate (Na_2SO_4, $10H_2O$) and 0·5 cc. 10 % sodium tungstate (Na_2WO_4, $2H_2O$), and 0·5 cc. 2/3 *N* sulphuric acid is added. The mixture is centrifuged or filtered; in either case the intact corpuscles are removed mechanically, being thrown down by the centrifuge, or, in the case of filtration, caught up in the precipitated plasma-proteins. For this reason less tungstic acid is required than in the ordinary Folin-Wu precipitation. The precipitate remains bright red, and microscopically the corpuscles are seen to be intact. The nitroprusside reaction of the filtrate is negative; therefore the filtrate contains no reduced glutathione.

Separation of the filtrate should be accomplished without undue delay (*e.g.* within 30 minutes of the addition of the tungstate and acid). The p_H of this "sulphate-tungstic" filtrate is approximately the same as that of the Folin-Wu filtrate.

If the tungstic acid is in excess of the amounts specified, it is only possible to obtain a glutathione-free filtrate if the preparation of the filtrate is completed within 4 minutes of making the mixture. After this time, the tungstic acid begins to attack the corpuscles with liberation of glutathione, as is shown by the gradual and parallel development of the following changes: the colour of the protein precipitate changes, as a result of the formation of acid haematin; microscopically, "ghosts" of corpuscles appear in the mixture; the filtrate gives a positive nitroprusside reaction of increasing intensity; and the apparent "sugar" figure of the filtrate, analysed by the ferricyanide method, rises.

Sodium sulphate is chosen for the isotonic solution because it does not interfere with the blue colour in the Folin-Wu and Benedict methods. Chloride causes a rapid fading of this colour; this has also been noted by Everett, Shoemaker, and Sheppard [1927].

In the above technique the glutathione is retained in the corpuscles. The glucose, on the other hand, diffuses freely throughout the precipitation mixture, as will be shown in considering the analytical results.

Note on a colour reaction of unknown origin.

While working out the new technique we observed a curious colour reaction. When blood was mixed with isotonic sodium chloride solution, an excess of tungstic acid added, the mixture centrifuged, and the supernatant fluid poured through a filter, the filter paper gradually developed a blue colour. This colour faded in the dark and reappeared on exposure of the paper to light; it was marked when 1 cc. 10 % sodium tungstate and 1 cc. 2/3 *N* sulphuric acid were used for 1 cc. blood, and very faint when the proportions were 0·5 cc. of each reagent for 1 cc. blood. We have not investigated the cause of this colour, but it is of interest that a similar colour appeared on the paper through which mixtures of yeast and tungstic acid were filtered. We never observed this colour when mixtures of yeast with blood or plasma were precipitated by tungstic acid; nor was it ever seen with the ordinary Folin-Wu precipitation of blood.

RESULTS.

The results of twelve experiments on six normal subjects are given in Table I. The salient points are as follows.

On *zinc hydroxide filtrates* (Somogyi's technique) all methods agree fairly well; the maximum variation is 9 mg. per 100 cc. There is a tendency for the two titration methods to give slightly higher figures than the colorimetric methods. On an average the Hagedorn-Jensen figure is 4 mg., the Shaffer-Hartmann, 5 mg., the Folin-Wu, 2 mg. per 100 cc. higher than the Benedict figure. The differences are small and it is impossible to say whether they are due to technical causes or to the presence in the zinc filtrates of traces of interfering substances. Somogyi [1929, 2] has applied various methods to zinc hydroxide filtrates and found that they agree within the limits of experimental error.

Table I.

Date and subject	Methods	Reducing substance expressed as mg. glucose per 100 cc. blood				
		Folin-Wu filtrate (*a*)	Sulphate-tungstic filtrate (*b*)	Somogyi's zinc hydroxide filtrate	Non-sugar effect (*a–b*)	True sugar
31. x. 29	Hagedorn-Jensen	105	76	76	29	74
K.	Shaffer-Hartmann	90	73	67	17	
	Folin-Wu	84	72	—	12	
	Benedict [1928]	74	72	—	2	
4. xi. 29	Hagedorn-Jensen	175	151	151	24	147
F.K.H.	Shaffer-Hartmann	165	145	144	20	
	Folin-Wu	156	142	—	14	
	Benedict [1928]	146	150	—	-4	
6. xi. 29	Hagedorn-Jensen	120	90	95	30	89
M.C.B.	Shaffer-Hartmann	109	92	90	17	
	Folin-Wu	97	87	—	10	
	Benedict [1928]	89	83	—	6	
15. xi. 29	Hagedorn-Jensen	86	62	60	24	60
K.	Shaffer-Hartmann	81	63	61	18	
	Folin-Wu	74	63	58	11	
	Benedict [1928]	57	57	57	0	
18. xi. 29	Hagedorn-Jensen	117	92	86	25	86
E.	Shaffer-Hartmann	109	92	89	17	
	Folin-Wu	99	90	81	9	
	Benedict [1928]	80	81	80	-1	
27. xi. 29	Hagedorn-Jensen	119	90	94	29	91
M.C.B.	Shaffer-Hartmann	113	94	92	19	
	Folin-Wu	104	91	90	13	
	Benedict [1928]	89	90	90	-1	
3. xii. 29	Hagedorn-Jensen	112	87	85	25	83
E.	Shaffer-Hartmann	109	87	87	22	
	Folin-Wu	100	81	82	19	
	Benedict [1928]	83	78	81	5	
6. xii. 29	Hagedorn-Jensen	119	87	83	32	83
G.A.H.	Shaffer-Hartmann	113	90	90	23	
	Folin-Wu	99	83	81	16	
	Benedict [1928]	78	79	78	-1	
16. xii. 29	Hagedorn-Jensen	97	72	67	25	70
R.H.	Shaffer-Hartmann	85	73	73	12	
	Folin-Wu	84	73	69	11	
	Benedict [1928]	70	68	69	1	
19. xii. 29	Hagedorn-Jensen	166	134	131	32	131
F.K.H.	Shaffer-Hartmann	152	130	128	22	
	Folin-Wu	153	137	135	16	
	Benedict [1928]	129	127	128	2	
23. xii. 29	Hagedorn-Jensen	133	107	106	26	101
R.H.	Shaffer-Hartmann	120	101	101	19	
	Folin-Wu	112	101	101	11	
	Benedict [1928]	96	93	100	3	
20. i. 30	Hagedorn-Jensen	118	85	85	33	81
G.A.H.	Shaffer-Hartmann	102	81	81	21	
	Folin-Wu	100	84	82	16	
	Benedict [1928]	79	75	79	4	

The "*sulphate-tungstic*" *filtrates* give values which are in good agreement with those found for the zinc hydroxide filtrates. All the methods applied to them agree fairly well; the variations are of the same order as those found for the zinc hydroxide filtrates, and may be due to the presence of traces of interfering substances or to technical causes.

In every experiment the *Folin-Wu filtrates* give higher figures than the zinc hydroxide and "sulphate-tungstic" filtrates, by every method except Benedict's. The highest values are always obtained by the Hagedorn-Jensen method, and after this in order come the Shaffer-Hartmann, Folin-Wu, and Benedict methods.

In any given experiment the *Benedict figure* is approximately the same for all three filtrates. The maximum variation is 7 mg. per 100 cc., and only two experiments show a greater discrepancy than 5 mg. per 100 cc. The variation is therefore within the limits of experimental error.

DISCUSSION.

True sugar.

For each blood sample we have nine determinations which we know are unaffected by glutathione. These include all determinations on the zinc hydroxide and "sulphate-tungstic" filtrates, since these filtrates contain no glutathione, and the determination on the Folin-Wu filtrate by the Benedict method, which is unaffected by the glutathione. These nine determinations agree fairly well; the maximum variation is 14 mg. per 100 cc., and in all but three experiments the figures fall within 10 mg. per 100 cc. Somogyi [1929, 1] has proved that his zinc hydroxide filtrates contain only fermentable sugar. Since in our experiments the "sulphate-tungstic" filtrates show the same figures as the zinc filtrates, we may regard these also as representing only true sugar. The possible criticism that in our method glucose might be retained in the corpuscles is also met by this agreement, since the corpuscles are laked in the zinc hydroxide technique. Also, the Benedict figure on the "sulphate-tungstic" filtrate agrees with the Benedict figure on the Folin-Wu filtrate; this indicates that both filtrates contain the same concentration of glucose.

The slight variation which occurs among the nine determinations under consideration may possibly be due to traces of interfering substances, but a consideration of individual experiments shows that the variation is not more than might be expected as experimental error. Therefore any or all of the nine figures mentioned may be taken as representing the true blood-sugar value, unaffected by glutathione or other reducing material. The average of the nine figures for each experiment is listed in the tables as "True sugar," and the variation from this average is never greater than ± 7 mg. per 100 cc., and is usually less than this.

Non-sugar effect.

The differences between the figures given by the Folin-Wu filtrate and those given by the "sulphate-tungstic" filtrate must be due entirely to the non-diffusible reducing substances derived from corpuscles. If the "sulphate-tungstic" filtrate may be regarded as giving true sugar figures, then the whole of the non-sugar reducing material must consist of the non-diffusible substances of corpuscles. The Folin-Wu filtrate contains this non-diffusible material, which must be partly or wholly accounted for as glutathione. The effect due to this (expressed as glucose) is determined, for each method, by subtracting the figure obtained with the "sulphate-tungstic" filtrate from that obtained with the Folin-Wu filtrate. These difference values are listed in the table under the heading "non-sugar effect." The amount indicated varies consistently with the method used, but for any one method a striking constancy is maintained through the series. These facts may be summarised as follows:

Method	Maximum Exp. 6. xii. 29 (mg. per 100 cc.)	Minimum Exp. 18. xi. 29 (mg. per 100 cc.)	Average (mg. per 100 cc.)
Hagedorn-Jensen	32	25	28
Shaffer-Hartmann	23	17	18
Folin-Wu	16	9	13
Benedict	-1	-1	1

When these effects observed in blood are compared with the effects produced by pure glutathione, it is found that for each experiment it is possible to explain the whole of the observed effect by postulating a definite concentration of glutathione in the blood. Thus, from the curves plotted for pure glutathione (Fig. 1 of the preceding paper) we can ascertain the effect, expressed as mg. glucose per 100 cc., that would be produced by various concentrations of reduced glutathione:

	59 mg. G.SH per 100 cc.	44 mg. G.SH per 100 cc.	52 mg. G.SH per 100 cc.
Hagedorn-Jensen	32	24	29
Shaffer-Hartmann	23	17	20
Folin-Wu	13	10	11
Benedict	0	0	0

The maximum non-sugar effect observed in the experiments on blood (Table I, G.A.H. 6. xii. 29) corresponds closely to the effect which would be produced by a blood-glutathione of 59 mg. per 100 cc.; the minimum effect (Table I, E. 18. xi. 29) may be explained on the hypothesis that that blood contained 44 mg. glutathione per 100 cc.; the average effect is very close to that produced by 52 mg. glutathione per 100 cc. Similarly for each individual blood sample it is possible to find a concentration of glutathione (between 44 and 59 mg. per 100 cc. blood), which will entirely explain the non-sugar effect and the discrepancies among the methods applied to Folin-Wu filtrates. The agreement among all methods applied to the other two filtrates is adequately explained by the fact that they contain no glutathione.

If the whole of the non-sugar effect is due to glutathione the concentration of this substance in normal human blood must be of the order of 52 mg. per

100 cc. The amount of glutathione in human blood has not yet been accurately determined. The published figures [Hunter and Eagles, 1927; Benedict and Newton, 1929, 1] indicate a concentration of 40 to 90 mg. per 100 cc. whole blood, but these figures rest on the assumption that glutathione is the only substance, present in the blood in appreciable amounts, which can give a positive nitroprusside reaction.

The evidence we have obtained is strongly suggestive that the non-diffusible reducing material, derived from corpuscles, and present in the Folin-Wu blood filtrates, consists entirely of glutathione, but it is possible that other substances may be included. However, any such substances, if they exist, must have several properties in common with glutathione. They must be non-diffusible and confined to corpuscles; they must be precipitated or destroyed in the zinc hydroxide precipitation; they must have no effect on the Benedict reagent, and must affect the Hagedorn-Jensen, Shaffer-Hartmann, and Folin-Wu reagents in the same ratio as does glutathione.

This particular numerical ratio may not be characteristic of glutathione only. It was pointed out, in the preceding paper, that the reducing action of glutathione expressed in terms of thiosulphate (cc. $N/200$) was almost the same in the Shaffer-Hartmann as in the Hagedorn-Jensen method; but since the amount of thiosulphate which corresponds to a given amount of glucose is not the same in the two methods, the effect of glutathione, expressed as glucose, is different in the two methods. All the methods of blood-sugar estimation are empirically calibrated for glucose under the standard conditions of the method, and the reduction produced by a given amount of glucose is not a constant quantity for all methods. Therefore, any non-glucose reducing substance which produced the same reduction (expressed in terms of hydrogen) in all the methods would necessarily show different glucose equivalents in the different methods. In the two titration methods used, a constant reduction effect would show itself as a constant relation to thiosulphate, the same for both. The glucose equivalents of any substance which behaved in this way in the Hagedorn-Jensen and Shaffer-Hartmann methods would be in approximately the same ratio as the glucose equivalents of glutathione. Unfortunately it is not possible to calculate what the effect would be in the Folin-Wu method; all that can be done in the colorimetric method is to compare the reducing action of the non-glucose substance with that of glucose.

Our conclusion must therefore be stated as follows. The discrepancies between the various blood-sugar methods, applied to different blood filtrates, can be explained by the single postulate that the blood contains a certain definite amount of glutathione, on an average, 52 mg. per 100 cc. whole blood, and figures of this order are supported by estimations depending on the nitroprusside reaction.

If it were independently proved that the concentration of glutathione in human blood falls within the narrow limits suggested by our figures, then the non-sugar reducing material of human blood would be completely accounted for.

Discussion of points in the literature in the light of the present findings.

The results of the present work confirm the main results of Herbert and Groen [1929] and support very strongly the suggestion then made, that glutathione is the main cause of the discrepancies between blood-sugar methods. Two minor inconsistencies between the present and former results call for mention. In the earlier paper the average discrepancy between the Folin-Wu and Shaffer-Hartmann methods was zero; in the present work the Shaffer-Hartmann figures are, on the average, 5 mg. per 100 cc. higher than the Folin-Wu figures. The hypothesis that glutathione is the main non-glucose reducing substance in blood demands a difference of 8 mg. per 100 cc. (for 50 mg. glutathione per 100 cc.). Secondly, in the earlier work the Benedict method (tungstic filtrates) gave higher figures than the Hagedorn-Jensen method (zinc filtrates); the average discrepancy was for plasma 9, for whole blood 10, and for corpuscles 10 mg. per 100 cc. It was realised that this could not be due to the blood-glutathione, since the difference was the same for plasma and for corpuscles. In the light of the present work it seems probable that there was some technical cause tending to raise the Benedict figures, though it cannot now be traced. The results of the present work accord with the known behaviour of glutathione.

MacLean's method must also be briefly considered. In the work of Herbert and Groen this method was shown to agree fairly well with the Hagedorn-Jensen method applied to Hagedorn-Jensen filtrates and to give figures lower than the Shaffer-Hartmann method by 7 mg. per 100 cc. on plasma, 20 mg. per 100 cc. on whole blood, and 39 mg. per 100 cc. on corpuscles. The relation of results by MacLean's method to results by other methods shows that MacLean's method gives figures close to the true sugar value, and in the earlier paper evidence was given that this was due to the use of ferric hydroxide filtrates, which contain little or no non-glucose material. We have been unable to determine how glutathione in blood would affect MacLean's method because the behaviour of pure glutathione was irregular both in the protein precipitation stage and in the copper reduction. Possibly the conditions of blood-protein precipitation are more favourable to the removal of glutathione than are the conditions in our artificial mixtures—for in the blood the glutathione is confined to corpuscles, whereas in our mixtures the glutathione was free in solution. From the practical standpoint, we may say that in ordinary blood analyses MacLean's method gives regular results which are close to the true sugar value.

Somogyi [1927, 1928, 1929, 1, 2; Somogyi and Kramer, 1928] has shown that the non-fermentable reducing substances of blood are present mainly in corpuscles, and are responsible for the discrepancies between certain blood-sugar methods applied to tungstic acid filtrates. He found no non-fermentable reducing substance in zinc hydroxide filtrates made by his technique, and the figures obtained by the Shaffer-Hartmann, Folin-Wu, Folin, and Benedict methods, applied to these filtrates, agreed within the limits of experimental error. The total reduction value of his zinc hydroxide filtrate was found to agree with the "fermentable sugar" in the Folin-Wu filtrate from the same blood. This agreement places the technique of true sugar determination in Folin-Wu filtrates by fermentation methods on a firmer basis, as the estimation of residual reduction was always open to the objection that the non-sugar reducing material might include glutathione derived from yeast. We have observed that glutathione is not removed from yeast by washing as in Somogyi's technique; the glutathione must have remained inside the yeast cells in Somogyi's experiments.

When calculating true sugar as the difference between the total reduction and the residual reduction after yeast fermentation, it should be remembered that, in some methods, non-sugar substances have a different glucose equivalent in the presence and in the absence of glucose. We find that, with the Hagedorn-Jensen ferricyanide reagent, glutathione has a higher glucose equivalent in the absence than in the presence of glucose, whereas in the Shaffer-Hartmann and Folin-Wu methods the glucose equivalent remains the same whether glucose is present or absent. Somogyi [1929, 2] has pointed out that the non-glucose reducing material of blood has a different effect on the Folin [1926] reagent in the presence and in the absence of glucose. Benedict [1928] states that his (1928) reagent is affected by the non-glucose reducing material of blood in the absence, but not in the presence, of glucose. Our study of the effect of pure glutathione on this reagent does not shed any light on this statement; our experience is that, in the absence of glucose, the effect of such quantities of glutathione as might be present in blood filtrates is negligible.

Our figures for different blood filtrates, as well as our observation that glutathione produces no measurable effect on the Benedict reagent, support Benedict's claim that his method gives true sugar figures on Folin-Wu filtrates.

Benedict and Newton [1929, 2] have adduced evidence that glutathione is the main non-glucose reducing substance in sheep's blood. They suggest that human blood contains at least one, and probably two, non-sugar reducing substances in addition to glutathione and ergothioneine, but, as far as we know, they have not yet published the evidence for this opinion.

As regards ergothioneine, the amount in tungstic acid filtrates from human blood is too small to have an appreciable effect on blood-sugar methods, as was pointed out by Herbert and Groen [1929]. Benedict and Newton [1929, 1] have found higher figures for ergothioneine in tungstomolybdic acid filtrates than in tungstic acid filtrates, but even with the tungstomolybdic acid method, the figures were below 10·1 mg. per 100 cc. (with the exception of a single sample with a figure of 24·4). It is our experience that concentrations below 10 mg. per 100 cc. would have very slight effects on blood-sugar analyses (less than 6 mg. per 100 cc., as glucose, by the ferricyanide method, which shows the greatest effect).

Sjollema [1927] determined the effect of glutathione on the Folin-Wu reagent and on ferricyanide. The ratio of glutathione to its glucose equivalent was 100 : 12·5 for the Folin-Wu reagent, and 100 : 48·5 for the ferricyanide method. He noted that oxidised glutathione had the same effect as the reduced form. Benedict and Newton [1929, 2] also noted that oxidised and reduced glutathione have the same effect on the Folin-Wu reagents. We have not studied the effect of oxidised glutathione, but we have shown that the reduction produced by reduced glutathione is due to some other change than the oxidation of the sulphydryl group. Benedict and Newton found that the ratio of glutathione to its glucose equivalent is 100 : 20 in the Folin-Wu method.

Evidence has been given in the present paper that the new "sulphate-tungstic" filtrate, like Somogyi's zinc hydroxide filtrate, gives true sugar figures for normal human blood. It must not be assumed *a priori* that these methods will universally give true sugar figures in other species, or in pathological conditions in man. It is necessary to consider each species separately. For example, Hiller, Linder and Van Slyke [1925] stated that the Hagedorn-Jensen method applied to tungstic filtrates gave the same figures as when zinc hydroxide filtrates were used. They used dog's blood, which, as Uyei [1926] has shown, contains about 26 mg. glutathione per 100 cc. corpuscles—an amount too small to affect appreciably the determinations on whole blood.

The difference between the figures obtained on the Folin-Wu and "sulphate-tungstic" filtrates is an index of the non-diffusible reducing material of corpuscles, which consists mainly, if not wholly, of glutathione. In normal human blood there is no appreciable amount of other, diffusible, non-sugar reducing substances, and the "sulphate-tungstic" filtrate gives true sugar figures. If, in other species, or in pathological conditions in man, there were any such diffusible reducing substances present, the "sulphate-tungstic" filtrate would not give true sugar figures, but the difference between the total reducing value of Folin-Wu and of "sulphate-tungstic" filtrates could still be used as a measure of the non-diffusible fraction of the non-glucose reducing material.

SUMMARY.

1. A modification of the Folin-Wu tungstic acid precipitation is described. The corpuscles are retained intact by the substitution of an isotonic sulphate solution for distilled water. The non-diffusible glutathione remains in the cells, while the glucose diffuses freely throughout the mixture.

2. In a series of experiments on normal human blood, the Folin-Wu filtrate, the "sulphate-tungstic" filtrate, and the Somogyi zinc hydroxide filtrate from the same blood were each analysed by four methods: (1) the ferricyanide method, (2) the Shaffer-Hartmann method, (3) the Folin-Wu method, and (4) the Benedict (1928) method.

3. The four methods agree when applied to the zinc hydroxide filtrates and to the "sulphate-tungstic" filtrates, which contain no glutathione. The Benedict method, which is unaffected by glutathione, gives the same figure for these

and for the Folin-Wu filtrate. All these determinations are believed to represent true sugar.

4. Definite non-sugar reducing effects are observed when the Hagedorn-Jensen, Shaffer-Hartmann, and Folin-Wu methods are applied to Folin-Wu tungstic acid filtrates. The average values of these effects, expressed as glucose, are for the ferricyanide method, 28, for the Shaffer-Hartmann method, 18, and for the Folin-Wu method, 13 mg. per 100 cc. The whole of this effect is shown to be due to the non-diffusible reducing material derived from corpuscles.

5. It is shown that the results may be explained by the single postulate that the blood analysed contained a certain definite quantity of reduced glutathione (44–60 mg. per 100 cc. whole blood), present in corpuscles in non-diffusible form. This hypothesis is supported by the published data on the concentration of glutathione in human blood.

6. Comparison of the zinc hydroxide precipitation method of Somogyi with that of Hagedorn and Jensen, using the ferricyanide method of analysis in each case, showed that the Hagedorn-Jensen filtrates gave slightly higher figures than the Somogyi filtrates. This may be explained by the known behaviour of glutathione in the two precipitation methods.

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XXXVII. ON THE CATALYSIS OF THE OXIDATION OF CYSTEINE AND THIOGLYCOLLIC ACID BY IRON AND COPPER.

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Mathews and Walker [1909] studied the apparent autoxidation of cysteine to cystine and showed the acceleration of the velocity of oxidation by very small amounts of iron, copper, and other metals, and the inhibition by small quantities of cyanide. Warburg and Sakuma [1923] further found that metallic catalysis accounted for practically the whole oxidation of cysteine in air, and that cysteine purified from metals was almost, if not entirely, non-autoxidisable in neutral solutions; later, Harrison [1924] confirmed this.

Harris [1922] investigated the complexes formed by cysteine with various metals and proposed a theory for the catalysis in which, in the case of iron say, a ferrous-cysteine complex is first formed, this is oxidised by air to a coloured ferric-cysteine which in turn is rapidly reduced again to ferrous-cysteine at the expense of cysteine, which is thereby oxidised to cystine. Recently Michaelis [1929], from the results of a detailed study of metallic complexes of cysteine, especially a stable cobaltous-cysteine, has put forward a theory of the exact nature of these metallic complexes and their function in the catalytic oxidation.

None of these authors, however, seems to have studied the kinetics of these catalytic oxidations except Mathews and Walker in observing the effect of hydrogen ion concentration. Dixon and Tunnicliffe [1923] noticed the autocatalytic nature of the aerobic and anaerobic oxidation of glutathione, cysteine, and thioglycollic acid, and showed that the disulphide group accelerates the rate of oxidation. This work appeared just before that of Warburg and Sakuma. Warburg and Sakuma's findings made it seem possible that the acceleration produced by adding —SS— compounds might not be due to the —SS— itself but to catalytic metals in the —SS— preparation. Harrison [1927], however, confirmed Dixon and Tunnicliffe's effect with metal-free preparations of disulphide compounds. This fact has not yet been very satisfactorily accounted for by a theory for the mechanism of the oxidation.

The observations to be reported in this paper show that the aerobic oxidation of —SH groups, in the presence of metallic ions, does not seem to fit in with any simple theory of catalysis.

EXPERIMENTAL.

The oxygen uptake curves were obtained, using the Barcroft differential manometer. The left-hand cups contained 3 cc. of water in all experiments, the right-hand cups containing a total of 3 cc. of the solution of which the oxygen uptake was being studied. All experiments were carried out at room temperature and at an initial p_H of 7·3, except where otherwise stated.

CYSTEINE.

Effect of phosphate buffer.

It was found that phosphate buffer solution has a large inhibiting effect on the velocity of oxygen uptake in presence of iron or copper. In Fig. 1 are shown the results of an experiment to illustrate this. The right-hand cups of four manometers each contained 8 mg. of cysteine hydrochloride at p_H 7·3, two in $M/20$ phosphate buffer and two in water. To one aqueous solution and to one buffer solution, 0·1 cc. of a solution containing 0·05 mg. of $CuSO_4, 5H_2O$ per cc. was added, and to the other two, 0·05 cc. of a fresh solution containing 0·5 mg. of $FeSO_4, 7H_2O$ per cc.

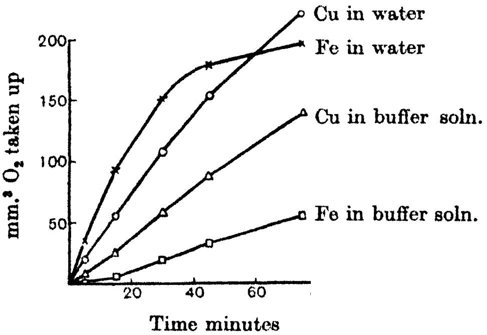


Fig. 1. Effect of phosphate buffer on oxygen uptake of cysteine solutions at p_H 7·3 in the presence of iron and copper.

The extent of the effect of phosphate buffer does not appear to vary much with the concentration between $M/20$ and $M/60$ phosphate.

As cysteine is quite an efficient buffer itself, most of the work was carried out using aqueous solutions of cysteine hydrochloride neutralised with N or $N/10$ sodium hydroxide to p_H 7·3. If the manometer cups are treated with acid and well washed out, there is usually little or no change in p_H at the end of the experiment, though the final liquid is occasionally slightly more alkaline than at first.

Effect of cysteine concentration.

The velocity of oxygen uptake is dependent, not only on the amount of catalyst present, but also upon the amount of cysteine. This was not made clear by Warburg and Sakuma, although Krebs [1929] noticed it in the case of haematin catalysis but not with pyridine-haematin or nicotine-haematin. That it is true in the case of copper and iron is shown in Figs. 2, 3 *b*, and 10. Fig. 2 records experiments carried out before the effects of phosphate buffer and high concentrations of copper (see below) were known. It was obtained by using a phosphate buffer solution of cysteine at p_H 7·3, to which copper solution was added. Different amounts of this mixture were put in the Barcroft cups and all made up to 3 cc. with buffer solution. The copper present was therefore actually increased proportionately with the cysteine; but in all cases the copper exceeded the minimum amount to give maximum effect. The curves *A*, *B*, and *C* are the results of three experiments, the velocities of uptake obtained being plotted against the amount of cysteine present; points on curve *B* have twice as much, and on curve *C* four times as much copper present as the corresponding points on curve *A*. Thus it is seen that the velocity of uptake is here dependent on the amount of cysteine present (and not on the amount of copper).

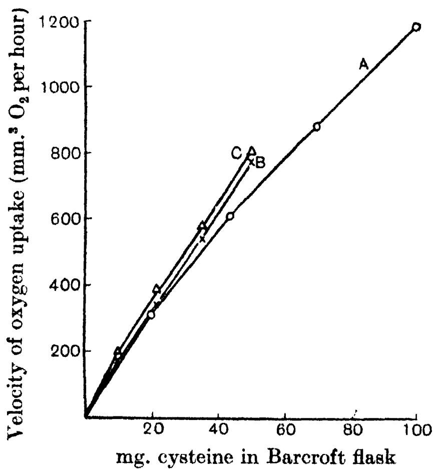


Fig. 2. Effect of cysteine concentration. Copper present in all in concentration more than sufficient to give maximum effect. Points on *B* have twice as much, points on *C* four times as much Cu as corresponding points on *A*.

The shape of the uptake curve.

There is also the curious fact that when the conditions are such that the velocity of uptake of 3 cc. is over about 400 mm.^{3} O_2 per hour, the uptake curve is almost invariably a straight line. That is, the velocity does not fall off as the unoxidised cysteine remaining in solution decreases, until it is completely oxidised, though the angle of the curve depends on the amount of cysteine originally present. Thus, in rapidly oxidising solutions, the velocity of oxygen uptake is dependent on the initial concentration of cysteine, but not on the concentration at any instant. At lower velocities there is generally a continuous falling off in rate of oxygen usage as the oxidation proceeds. Figs. 6 *a*, 7 *a*, and 11 show these effects.

The uptake curve is not autocatalytic in shape, and Dixon and Tunnicliffe [1923] considered this due to the insolubility of the disulphide form, cystine, which in separating out can have no effect on the course of the reaction. However, it has been noticed that at the end of the majority of the experiments, very little or no cystine had precipitated, and the precipitate developed slowly.

It was thought possible that the proportionality of reaction velocity to the initial concentration of cysteine might be explained by presuming that the cysteine hydrochloride used might contain a second substance which acted in a complementary way towards the metal as catalyst. But this could not be proved, though the following experiments were carried out to test it.

Various pure specimens of cysteine hydrochloride, including Kahlbaum's "Warburg" cysteine hydrochloride, were tried and found to give the same results. A sample of cysteine was purified by precipitation as the cuprous compound in the manner described by Hopkins [1929] for the separation of glutathione. 60 mg. of cysteine hydrochloride, thus purified and recrystallised from pure HCl solution, were made up to 15 cc. at p_H 7·3 with water and $N/10$ NaOH. Two Barcroft cups received 1·5 cc. of this (*i.e.* 6 mg. cysteine hydrochloride) and 1·5 cc. water, and two received 3 cc. of the solution. To one of each was added 0·033 cc. of a fresh solution containing 0·5 mg. $FeSO_4$, $7H_2O$ per cc., and to each of the others 0·1 cc. of a solution containing 1 mg. per cc. The initial velocities of O_2 uptake obtained with these are plotted in Fig. 3 *a* against the amount of iron present, and in Fig. 3 *b* against the cysteine-HCl content.

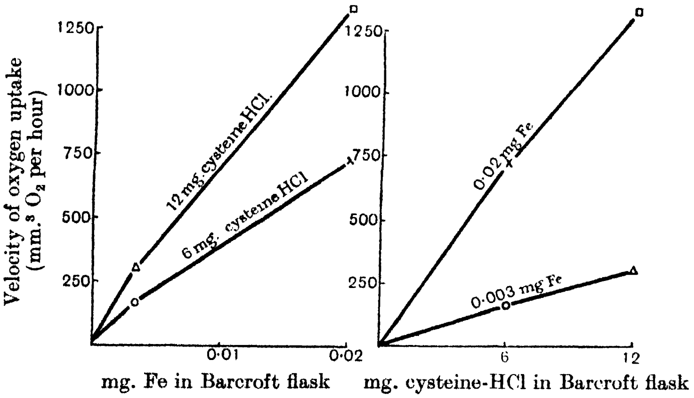


Fig. 3 *a*. Fig. 3 *b*.

Effects of changing iron and cysteine concentrations on the velocity of O_2 uptake.

It is seen that this behaved in the usual way, the velocity of O_2 uptake being proportional both to the cysteine and the iron present. The other samples of cysteine were all tested in this manner, similar curves being obtained if small amounts of copper were used instead of iron. A sample of optically inactive cysteine hydrochloride (obtained by boiling cystine in acid for a long time, and then reducing), kindly given me by Messrs N. W. Pirie and A. Leese, gave the same results. As the addition of sodium chloride had no effect on the course of the reaction, the chloride and sodium ions present in neutralised cysteine hydrochloride solutions evidently have no effect. An attempt was made to concentrate the possible catalytic complement in the following way. 0·25 g. of cysteine hydrochloride was made up to 20 cc. at p_H 7·3 in water. A small amount of copper (0·1 cc. of a solution containing 0·5 mg. $CuSO_4, 5H_2O$ per cc.) was added and the liquid aerated until completely oxidised, and then concentrated by distillation *in vacuo* to 4 cc. and filtered. (A separate bulk experiment with ten times the quantities but the same proportions showed that only about one-third of the copper remained in the filtrate, the rest being in the cystine precipitate.) Portions of this extract were added to solutions of cysteine containing copper or iron, but no acceleration of the oxygen usage was observed.

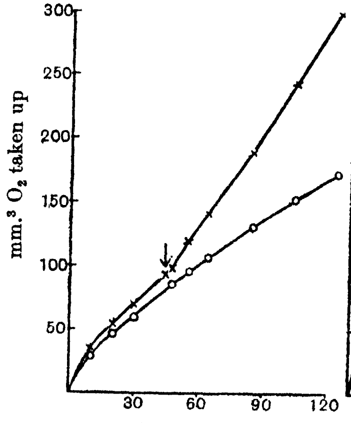


Fig. 4 *a*. Effect of adding extra cysteine during oxidation in presence of iron.

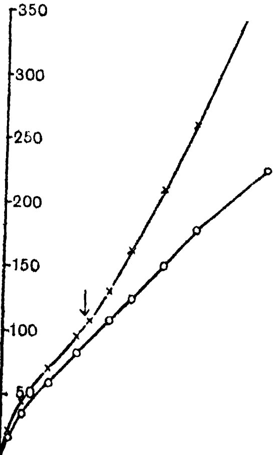


Fig. 4 *b*. Effect of adding extra cysteine during oxidation in presence of copper.

The effect of cysteine concentration was further studied by adding extra cysteine during the course of the oxidation, and when the oxidation was complete. The right-hand cups of two manometers contained respectively 2 cc. and 2·5 cc. of water, and each received 0·5 cc. of an aqueous solution containing 6 mg. of cysteine-HCl at p_H 7·4. In the first an extra 0·5 cc. of this solution was contained in a small tube suspended from the potash tube in the middle of the cup in the manner described by Keilin [1929]. To each cup was added 0·05 cc. of a fresh solution containing 0·2 mg. $FeSO_4, 7H_2O$ per cc. The oxygen uptakes obtained with these are shown in Fig. 4 *a*. At the point marked with an arrow, the one manometer was tapped so that the extra cysteine in the "Keilin tube" was added to the solution. Fig. 4 *b* was obtained in the same way, but the 0·5 cc. cysteine solution contained 8 mg. cysteine-HCl, and 0·1 cc. of a solution containing 0·5 mg. $CuSO_4, 5H_2O$ replaced the iron solution.

It is seen that on adding fresh cysteine solution to a solution in which cysteine has been partly oxidised in the presence of iron or copper, the oxygen uptake velocity increases, the new velocity of uptake being about what would be expected from the sum of the two amounts of cysteine.

Next, a manometer was set up having in the right-hand cup 1 cc. of an aqueous solution containing 4 mg. cysteine-HCl at p_H 7·3, and 0·05 cc. of a solution of 0·5 mg. $FeSO_4, 7H_2O$ per cc. It was shaken until the oxygen uptake had ceased. Then a further 1 cc. of the cysteine solution was added and the O_2 uptake observed. A second manometer contained in its right-hand cup, 2 cc. H_2O, 1 cc. of the cysteine solution and the same amount of iron as before, and its O_2 uptake was compared with the first. The uptake curves obtained are shown in Fig. 5 *a*, and it is seen that the presence of oxidised cysteine in the first manometer has practically no effect on the O_2 uptake.

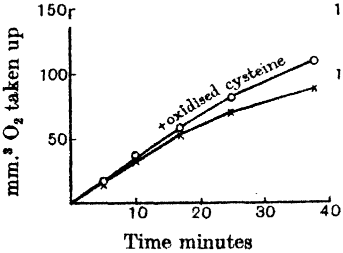


Fig. 5 *a*. Effect of the presence of previously oxidised cysteine on the oxidation of fresh cysteine. Iron catalyst.

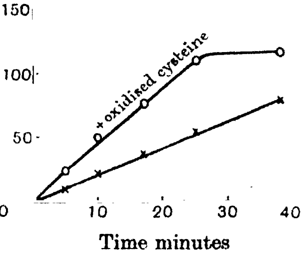


Fig. 5 *b*. Effect of the presence of previously oxidised cysteine on the oxidation of fresh cysteine. Copper catalyst.

Fig. 5 *b* shows the result of a similar experiment to the above, except that the iron solution was replaced by 0·1 cc. of a solution containing 1 mg. $CuSO_4, 5H_2O$ per cc. Here it is seen that in the presence of oxidised cysteine, the rate of O_2 uptake is about what would be expected if the whole of the cysteine had been added at once.

Thus in the presence of iron and completely oxidised cysteine, addition of fresh cysteine causes oxygen uptake to recommence at a velocity to be expected from the fresh cysteine alone, but with copper, the velocity is again what would be expected from the sum of the oxidised and fresh cysteine. This result has been repeatedly obtained.

Concentration of catalyst.

Harrison [1924] showed that the velocity of oxygen uptake of cysteine in the presence of iron is proportional to the amount of iron present up to a concentration of $1·6 \times 10^{-3}$ mg. of iron in 3 cc. The following experiment was made in order to see whether there was a limit to this proportionality. The right-hand cups of four manometers each contained 8 mg. cysteine-HCl in 3 cc. water at

p_H 7·3, and they received respectively the following amounts of iron: 0·1 cc. of a solution containing 0·05 mg. $FeSO_4, 7H_2O$ per cc.; 0·03 cc. of 0·5 mg. per cc.; 0·1 cc. of 1 mg. per cc.; and 0·5 cc. of 5 mg. per cc. The uptake curves obtained with these increasing amounts of iron are shown in Fig. 6 *a*, and in Fig. 6 *b* the velocities of O_2 uptake are plotted against the amounts of iron.

It is thus seen that the velocity of oxygen uptake is proportional to the amount of iron at least up to a concentration of iron of 0·05 mg. per cc.

With copper the case is very different. Up to a certain small concentration, the uptake velocity is proportional to the copper, but at higher copper concentrations the velocity/copper curve falls off sharply, becoming horizontal, and any further increase in copper concentration causes no further increase in reaction velocity. In unbuffered solutions a very large amount of copper added actually causes the velocity to decrease again slightly. This decrease is, however, explained by the fact that on adding a fair amount of copper sulphate, an insoluble Cu-cysteine precipitate is formed, leaving H_2SO_4 in solution so that the p_H is lowered. Smaller additions of copper sulphate cause no appreciable change in p_H.

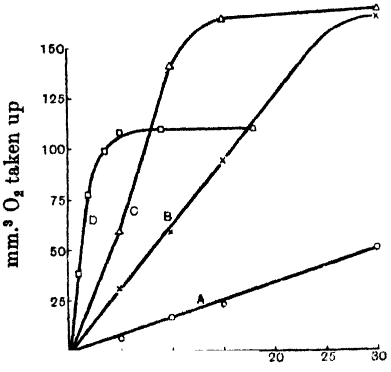


Fig. 6*a*. Oxygen uptake curves given by 8 mg. cysteine hydrochloride in water at p_H 7·3 in presence of:
(*A*) 0·0011 mg. Fe, (*B*) 0·0033 mg. Fe,
(*C*) 0·022 mg. Fe, (*D*) 0·054 mg. Fe.

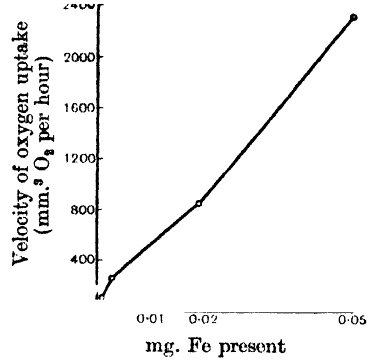


Fig. 6*b*[1]. Effect of increasing iron on the velocity of oxidation of 8 mg. cysteine hydrochloride at p_H 7·3.

The following is a typical experiment illustrating this effect. The right-hand cups of eight manometers, each containing 8 mg. of cysteine hydrochloride in 3 cc. water at p_H 7·3, received amounts of copper increasing from 0·05 cc. of a solution containing 0·05 mg. $CuSO_4, 5H_2O$ per cc., to 0·2 cc. of a solution containing 1 mg. $CuSO_4, 5H_2O$ per cc., and a ninth received no added metal. The uptake curves given by these are shown in Fig. 7 *a*, and Fig. 7 *b* is obtained by plotting initial velocities against copper present.

The failure of the velocity of O_2 uptake to increase with copper concentration cannot be explained by oxygen lack in the liquid, as with iron, and in

[1] The upper points on this curve should actually be higher, as the rate of shaking during the experiment was afterwards found not to be sufficiently great to eliminate all diffusion effect at these high velocities.

other experiments shown, much higher velocities were obtained under the same conditions of temperature and rate of shaking.

This minimal concentration of copper for maximal effect is not greatly affected by temperature, change in cysteine concentration or oxygen tension, the presence or absence of phosphate buffer, by increasing the liquid-glass surface by the introduction of glass beads, or by change of hydrogen ion concentration between p_H 6 and 8.

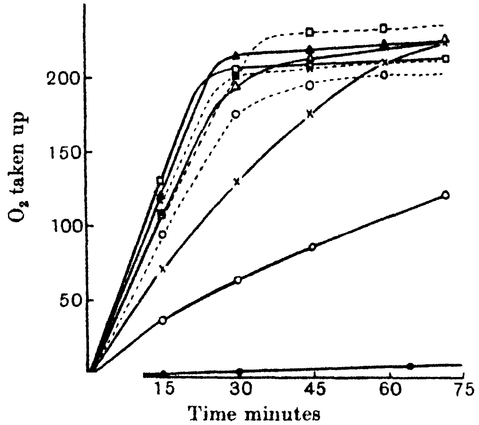


Fig. 7*a*. Oxygen uptake curves given by 8 mg. cysteine hydrochloride in water at p_H 7·3 in presence of:

——○—— 0·00064 mg. Cu.
——△—— 0·0042 mg. Cu.
- - -○- - - 0·0038 mg. Cu.
- - -▲- - - 0·0256 mg. Cu.
——●—— No added metal.
——×—— 0·00128 mg. Cu.
——□—— 0·017 mg. Cu.
- - -×- - - 0·0128 mg. Cu.
- - -□- - - 0·051 mg. Cu.

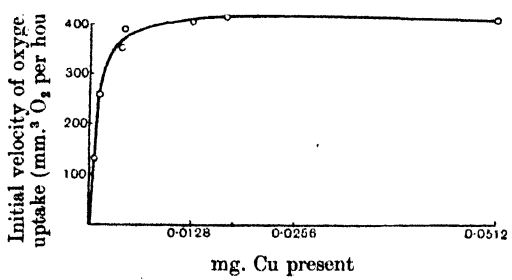


Fig. 7*b*. Effect of increasing amounts of copper on the velocity of oxidation of 8 mg. cysteine hydrochloride at p_H 7·3.

In Fig. 8 is given a series of curves obtained under various conditions, which show the effect of increasing copper concentration.

A converse effect with copper occurs in high concentrations of cysteine with low copper. If increasing amounts of cysteine are put up with a constant very small amount of copper, the oxygen uptake velocities are at first proportional to the amount of cysteine, and then a further increase in cysteine concentration causes scarcely any increase in uptake velocity. If now the copper is increased, the proportionality to cysteine continues to a higher level before ceasing. Fig. 9 illustrates these observations, and it was obtained in the following manner. A solution of 250 mg. cysteine-HCl in 15 cc. water at p_H 7·3 was made up, and the right-hand cups of four manometers received respectively 3 cc., 2·2 cc., 1·2 cc., and 0·4 cc. of this. The volume in each was made up to 3 cc. with water, and to each was added 0·05 cc. of a solution containing 0·05 mg. $CuSO_4$, $5H_2O$ per cc. The initial velocities of O_2 uptake obtained from these are shown in the lower curve in Fig. 9, plotted against the amount of cysteine-HCl present. The upper curve is the result of an exactly similar experiment, using just double the amount of copper solution.

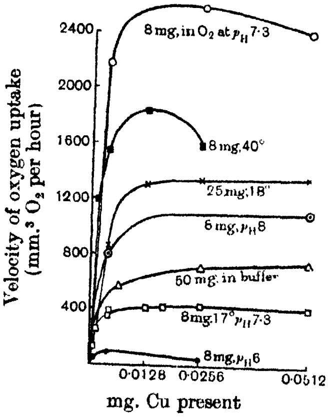


Fig. 8. Effect of increasing amounts of copper on the velocity of oxidation of cysteine under various conditions.

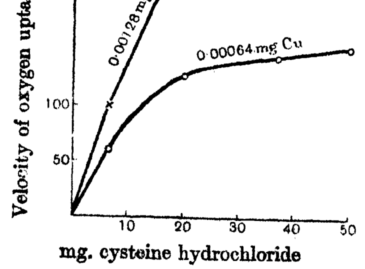


Fig. 9. Effect of high concentration of cysteine on the velocity of oxygen uptake in presence of very small amounts of copper.

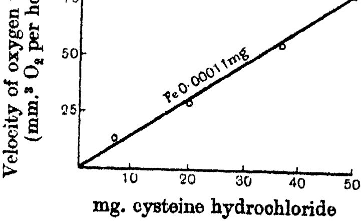


Fig. 10. Effect of high cysteine concentration on the velocity of oxygen uptake in presence of a small amount of iron.

In the case of iron catalysis, in the presence of a very small amount of iron, the uptake velocity with large amounts of cysteine remains proportional to the cysteine as seen in Fig. 10, which was obtained in the same way as Fig. 9, the copper solution being replaced by 0·1 cc. of a solution containing 0·05 mg. $FeSO_4, 7H_2O$ per cc.

Oxygen tension.

The right-hand cups of four manometers, *A*, *B*, *C*, and *D*, each contained 10 mg. cysteine-HCl in 2·5 cc. water at p_H 7·3. "Keilin tubes" were suspended in all, those in *A* and *C* each holding 0·05 cc. of a solution containing 0·5 mg. $FeSO_4, 7H_2O$ per cc., plus 0·5 cc. water, and those in *B* and *D* having 0·1 cc. of a solution containing 0·05 mg. $CuSO_4, 5H_2O$ per cc., plus 0·5 cc. water. *C* and *D* were evacuated and filled with oxygen at atmospheric pressure. The oxygen uptake in all was noted, and after 21 minutes all were tapped so that the iron and copper solutions became mixed with the cysteine, and the new uptakes were observed. The results of this experiment, which are seen in Fig. 11, show that increasing the oxygen tension causes a very considerable increase in the rate of cysteine oxidation in the presence of either iron or copper, though the velocity does not appear to be proportional to the partial pressure of the oxygen in the gas. Similar results were obtained by Krebs [1929] with haematin or nicotine-haematin as catalyst.

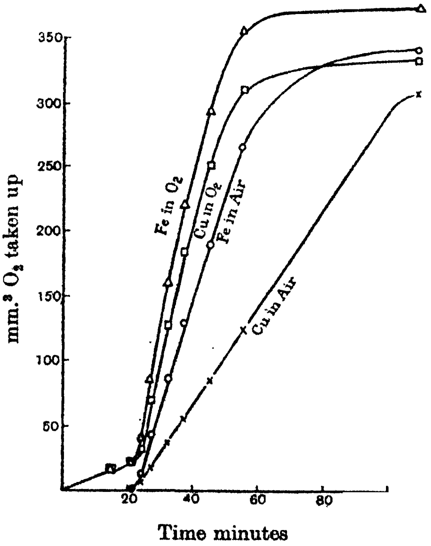


Fig. 11. Effect of increased oxygen tension on the velocity of oxygen uptake of cysteine in presence of iron and copper. Cu or Fe solutions added 22 minutes from start.

THIOGLYCOLLIC ACID.

The acid used for these experiments was distilled *in vacuo*, using vessels that had been previously well boiled out with pure hydrochloric acid solution.

Phosphate buffer.

Phosphate buffer has a considerable inhibiting effect on the velocity of oxidation, which does not vary much between phosphate concentrations of *M*/20 and *M*/60. But, since thioglycollic acid has no buffering properties, it was necessary to use the buffer solution, as without it the solutions became acid on oxidation.

Oxygen uptake curve.

With thioglycollic acid the autocatalytic acceleration, noted by Dixon and Tunnicliffe, is obtained, but, as with cysteine at high velocities of uptake, the velocity does not fall off until oxidation is complete (see Fig. 12). In the case of copper catalysis the initial velocity of uptake depends under the usual conditions on the amount of thioglycollic acid present. With iron this is possibly masked by another phenomenon described in the next section.

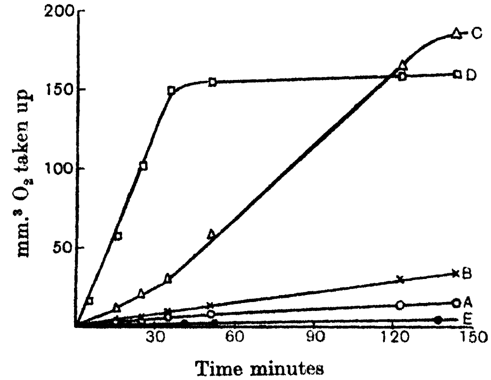


Fig. 12. Oxygen uptake curves given by 3·5 mg. thioglycollic acid in *M*/60 phosphate buffer at p_H 7·3 in presence of:

(*A*) 0·0054 mg. Fe. (*B*) 0·011 mg. Fe. (*C*) 0·022 mg. Fe. (*D*) 0·044 mg. Fe. (*E*) No added metal.

Concentration of catalyst.

When iron is the catalyst, the behaviour of thioglycollic acid is very different from that of cysteine. In this case we have a type of auto-inhibition, the catalyst is largely inactivated by excess of thioglycollic acid. The following experiments show this. 280 mg. thioglycollic acid were made up to 80 cc. at p_H 7·3 with *M*/20 phosphate buffer solution. The right-hand cups of four manometers each received 1 cc. of this and 2 cc. of water, and the following different amounts of iron were added: 0·05 cc. of a solution containing 0·5 mg. $FeSO_4, 7H_2O$ per cc.; 0·1 cc. of the same; 0·1 cc. of a solution of double that strength; and 0·2 cc. of the second solution. A control was also run containing no added metal. Fig. 12 gives the uptake curves thus obtained.

The upper curve in Fig. 13 was obtained by plotting the velocities of O_2 uptake, given by the above experiment, against the iron concentration. The two lower curves in Fig. 13 were obtained in a similar way, the one being the result of using 3 cc. of the same thioglycollic solution (and no water), and the other using 3 cc. of a thioglycollic acid solution of the same strength but only one-third the concentration of phosphate buffer.

It is seen that the first additions of iron cause very little increase in velocity, but after a certain concentration of iron is reached, the velocity increases rapidly, and the increase is proportional to the extra iron added. Where the thioglycollic acid is present in three times the strength, it is seen that not only is more iron required before its effect is appreciable, but also the velocity never increases, with increasing iron, at such a rate as in the case of the weaker solution. Velocity/iron curves of the same type, for the two different concentrations of thioglycollic acid, were obtained by using neutral water solutions without buffer; the velocities obtained were all considerably higher but the shapes of the curves were exactly the same as in the presence of phosphate buffer.

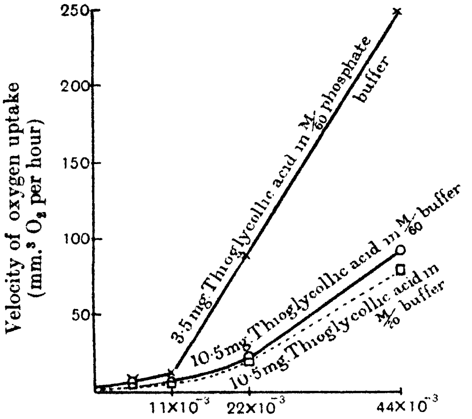


Fig. 13. Effect of increasing amounts of iron on the velocity of oxygen uptake of thioglycollic acid solutions at p_H 7·3.

It was thought that this inhibition might possibly be due to the presence of H_2S in the distilled thioglycollic acid, but it was actually found that with thioglycollic acid and iron, some acceleration of the reaction was produced by adding small quantities of Na_2S solution (though the p_H was not affected). Krebs [1929] has found the same thing with cysteine oxidation in presence of iron or nicotine-haematin.

With thioglycollic acid, similar curves of copper concentration against velocity of O_2 uptake are obtained as with cysteine; *i.e.* a concentration of copper is reached when further addition of copper gives no increase in velocity. The minimum copper for maximum effect is just about the same as for cysteine. This is seen in Fig. 14, which was given by the following experiment. 280 mg. of thioglycollic acid were made up to 80 cc. at p_H 7·3 with $M/60$ phosphate buffer solution. The right-hand cups of four manometers each received 1 cc. of this and 2 cc. of $M/60$ buffer. To the cups were added the following amounts of copper: 0·1 cc. of a solution containing 0·05 mg. $CuSO_4, 5H_2O$ per cc.; 0·2 cc. of the same; 0·1 cc. of a solution containing 0·25 mg. per cc.; and 0·2 cc. of a solution containing 0·5 mg. per cc. The lower curve in Fig. 14 shows the velocities of O_2 uptake obtained, plotted against the amount of copper present. The upper curve is the result of a similar experiment in which 3 cc. of the thioglycollic acid solution were used.

It will be noticed that in the curve obtained for the higher concentration of thioglycollic acid, the velocity corresponding to a very low copper concentration falls very low. This low point could be repeatedly obtained and corresponds with either the low oxygen uptake of excess of cysteine in presence of low copper (Fig. 9), or the auto-inhibition of thioglycollic acid on the oxidation with iron as catalyst.

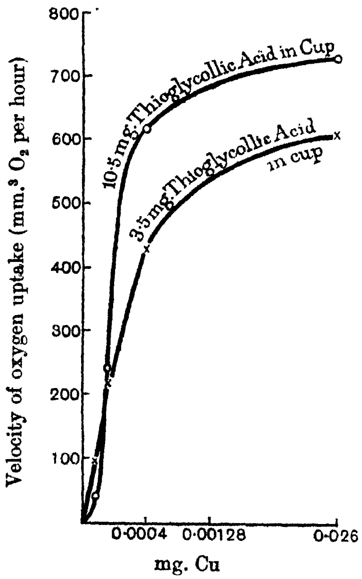


Fig. 14. Effect of increasing amounts of copper on the velocity of oxygen uptake of thioglycollic acid solutions at p_H 7·3.

Complexes of thioglycollic acid with iron and copper.

Harris [1922] described the complexes formed between cysteine and iron, copper, and other metals, and Hopkins [1929] described the cuprous compound of glutathione. Thioglycollic acid forms a similar series of complexes. The cuprous compound formed with cuprous oxide is white and resembles those of glutathione and cysteine in being readily soluble in strong HCl or in alkaline solution but insoluble in neutral or H_2SO_4 solution. On adding cupric sulphate to a neutral thioglycollic acid solution, a brown precipitate is momentarily formed and disappears immediately; this is probably a cupric thioglycollic acid compound, which is rapidly reduced to the cuprous compound, and corresponds to the blue-black momentary precipitate noticed in the case of cysteine.

On adding an iron salt to neutral thioglycollic acid solutions, a purple colour appears which is presumably the ferric complex of thioglycollic acid, as it fades on standing and reappears on aerating, thus corresponding to the violet colour obtained with cysteine. But here there is a noteworthy deviation from correspondence with cysteine. Whereas, as Harris showed, the rate of disappearance of the violet ferric cysteine colour varies directly with the concentration of cysteine, the rate of disappearance of the purple ferric thioglycollic compound is retarded very greatly by increasing the concentration of thioglycollic acid. This fits in with the observation mentioned above, that the aerobic oxidation of thioglycollic acid in the presence of iron is retarded by increasing thioglycollic acid concentration.

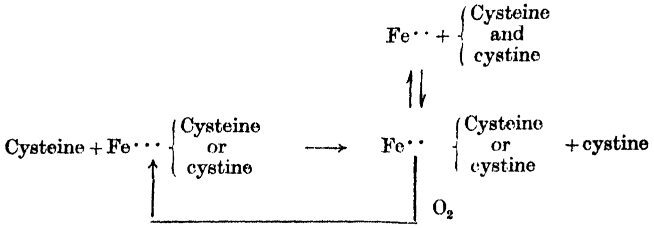
DISCUSSION.

The results reported in this paper show that the oxidation of sulphydryl compounds in presence of metals is not to be accounted for on any classical theory of catalysis. It has been found possible to make a fairly simple hypothesis, involving only principal valencies, which will fit the facts more or less satisfactorily; but considering Michaelis's [1929] view of the complex nature of the metallo-cysteine compounds, it would be unwise to suggest more than one or two of the main ideas on which the hypothesis is based.

It is somewhat difficult to decide what kinetic relations Michaelis's series of reactions would imply, but they do not seem to suggest the facts mentioned in this paper; namely, that the velocity of oxygen uptake of a given volume of solution should be proportional to the amount of metal present, and to the initial concentration of cysteine at high velocities, and also dependent on the oxygen tension.

It is tentatively suggested that the active catalyst may be considered to be not merely the metallic ion, but a reversible monomolecular combination between the metal and cysteine or cystine. This complex is then alternately oxidised by O_2 to the ferric state and reduced by cysteine back to the ferrous state as Harris [1922] showed. If the reduction process at p_H 7·3 is very rapid compared with the oxidation process, then the oxidation process would limit the reaction, and the velocity of oxygen uptake should be dependent on the O_2 tension, and on the amount of catalytic complex, which in turn, as it is reversibly formed, is proportional to both the iron and [cysteine + cystine] concentrations. As oxidation proceeds and cysteine is replaced by cystine, the amount of catalyst remains constant and the velocity does not fall until nearly all the cysteine has disappeared.

Making no assumptions as to the type of valencies, the system may be represented as follows:


Fe·· + Cysteine and cystine
Cysteine + Fe··· Cysteine or cystine
Fe·· Cysteine or cystine + cystine
O₂

A similar mechanism is suggested for copper catalysis. The metal-cysteine compounds might be formed by co-ordinate valencies, or they might be carboxylic salts, but could not, on this hypothesis, depend on the —SH group if they are to be formed with equal facility by the oxidised and the reduced forms.

The hypothesis requires that the cystine should not be removed by precipitation, and it has been noticed that at the end of the majority of experiments, there was either very little or no precipitate visible, but that it appeared in considerable amount after some time. This precipitation might therefore account for the falling off of the velocity in experiments in which the oxidation was proceeding more slowly.

The fact that, after a certain concentration is reached, the addition of further copper has no effect on the velocity of reaction may be due to the insolubility of copper compounds of cysteine or cystine and thioglycollic acid. A method for estimating cystine is actually based on this insolubility [Harris, 1923].

When the copper concentration was very low, it was observed that a concentration of cysteine was reached, after which further additions of cysteine caused very little increase in the rate of oxygen usage (Fig. 9). It is possible that the reaction between copper and cysteine, to give the catalytic complex, may have its equilibrium very much in favour of the catalytic complex, and in high cysteine concentration and very low copper practically all the copper may be used up, increase of cysteine then producing very little more of the catalytic complex.

To attempt to explain the p_H curve of Mathews and Walker, and the other phenomena observed in this paper, on this hypothesis might be carrying speculation too far. Such ideas as a tendency to hydrolysis of the metallo-complex in acid solutions, and formation of inactive salts in alkaline solutions, combination of phosphate, in phosphate buffer, with the metal or complex to give inactive substances, and different degrees of stability of the complex in solution to explain the different effects of previously oxidised cysteine in the cases of iron and copper, might be put forward in the absence of an explanation based on direct evidence.

A similar hypothesis might be applied to the case of thioglycollic acid oxidation; the auto-inhibitory effect of higher concentrations of thioglycollic acid with iron could possibly be explained by the formation of an inactive metallic complex containing more molecules of thioglycollic acid than the active catalyst.

The hypothesis readily explains the catalytic effect of added disulphide compounds; the autocatalytic effect, which has been observed by other authors, is not noticeable in cysteine oxidation at p_H 7·3, but appears to some extent in thioglycollic acid oxidation; it can only be explained on this hypothesis by the assumption that the catalytic metallic complex is more readily formed with a disulphide acid than with a sulphydryl acid.

Since this work was done, a paper has appeared by Cannan and Richardson [1929] on other aspects of the same subject. They have also put forward certain hypotheses for the mechanism of sulphydryl oxidation; but it is unfortunate that these have been generalised from a study of the complexes of thioglycollic acid with iron, since it has been shown above (see Fig. 13 and the section on metallic complexes) that thioglycollic acid and iron behave in an anomalous way.

SUMMARY.

1. The rate of oxidation of cysteine at p_H 7·3 is proportional to the amount of iron present up to the highest concentration tried.

2. It is proportional to the amount of copper present up to a certain small concentration. Further increase of copper, provided it does not affect the p_H, causes no effect. This minimal concentration of copper, giving maximal effect, does not appear to be greatly affected by change in temperature, oxygen tension, p_H, cysteine concentration, or by the presence of phosphate buffer or increased liquid-glass interface.

3. The velocity of oxygen uptake is also proportional to the amount of cysteine originally present.

4. The oxygen uptake curve is linear when the velocity of uptake is fairly rapid; it tends to fall off when the reaction is not so rapid.

5. In presence of very low concentrations of copper, a concentration of cysteine can be reached beyond which further additions of cysteine cause only slight increase in velocity.

6. Addition of an extra amount of cysteine during the course of the oxidation of cysteine, causes the velocity to increase to the rate that would be expected if both amounts of cysteine had been added at first, in the case of both iron and copper catalysis. If, however, the extra cysteine is added when the oxidation of the first lot is complete, the O_2 uptake recommences, when copper is the catalyst, at a rate to be expected from the total cysteine, while, in the case of iron, the new velocity is only what would be expected from the fresh cysteine.

7. The velocity of oxidation in presence of iron or copper is increased by increasing the oxygen tension.

8. The velocity of oxygen uptake in presence of iron or copper is decreased by the presence of phosphate buffer.

9. Thioglycollic acid in solution at p_H 7·3 shows similar behaviour to the above, except that:

(*a*) the velocity of O_2 uptake in the presence of iron is very low up to a certain concentration of iron, and thereafter increases rapidly in proportion to the amount of extra iron added;

(*b*) higher concentration of thioglycollic acid decreases the rate of oxygen uptake in the presence of iron.

10. A *résumé* of the behaviour of complexes of iron and copper with thioglycollic acid is given.

11. The above phenomena are discussed in relation to the type of hypothesis that may be necessary to correlate them.

I wish to thank Dr M. Dixon for his encouragement and criticism.

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XXXVIII. A STUDY OF THE COMPOSITION OF HUMAN MILK IN THE LATER PERIODS OF LACTATION AND A COMPARISON WITH THAT OF EARLY MILK.

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A PREVIOUS investigation gave the variations in the chemical composition of normal human colostrum and early milk [Lowenfeld, Widdows, Bond and Taylor, 1927]. This work has now been extended to a study of the composition of milk secreted after the first month of lactation with the view not only of comparing the relative quantities of the constituents at the different stages of lactation, but also of comparing the relative quantities of these substances at the same stage of lactation. The material for this work has been obtained from the children's department of the Royal Free Hospital, Shoreditch Carnegie Welfare Settlement and from the Mothercraft Training Society, Cromwell House, Highgate.

In the case of the analyses of the later milk it has not been found possible to deal with individual women over the whole course of the observations as was done in the work on early milk, although in some cases it has been managed over two or three months. By studying the composition of the milk of as large a number of women as possible it was thought that to a large extent individual differences would be eliminated and some generalisations indicated.

Altogether 51 women have been studied, and of these 34 paid single, 12 double and 5 triple visits to the Hospital.

The constituents of the milk investigated are sugar, protein, fat, calcium, phosphorus and ash. The methods employed in the estimation of these substances were the same as those for early milk. The number of estimations of each constituent has varied between 50 and 60. The method of extracting the milk was either by means of the breast pump or digital expression. The method of extraction is only of importance in dealing with the percentages of fat, also it was only in the estimation of the fat that separate estimations were made on the milk extracted before and after the baby had fed. The earlier work showed that in the case of the other constituents the differences in the fore and after milk were slight and of no particular significance. Therefore in this work, except in the case of the fat, the estimations were done on the mixed fore and after milk. The phosphorus, which was not studied in the earlier work, has been estimated by the Martland and Robison [1924] modification, of the Bell-Doisy [1920] and Briggs [1922] method, using Merck's perhydrol as suggested by them and Briggs for the final oxidation of the material for the total phosphorus present.

In considering the results of this work the calcium and phosphorus figures will first be considered separately and then their relationship to the ash and to one another.

The significance of the nitrogen and sugar results will also be dealt with, but the fat results will be left to another communication.

CALCIUM.

The calcium content of the milk of 51 women at all stages in the course of lactation has been determined with a view to ascertaining what variations exist, and whether, if there is variation, the variation follows any general rule. It has been emphasised by us [1927] and also by Hunnaeus [1909] that the individual differences in the calcium content of milk are much greater than the variations from day to day in any one individual. Samples of milk have been analysed, extending from the first to the tenth month of lactation, and the individual variation has again been found to be large, varying from 0·036 to 0·075 g. per 100 cc. of milk.

As our former work showed that the variations in calcium expressed themselves slowly, with the exception of the first month, the averages have been taken over a range of four months. The results of our work are shown on the left of Table I.

Table I.

No. of samples	Average CaO (g. per 100 cc. of milk)	Period of lactation	No. of samples	Average CaO (g. per 100 cc. of milk)	Period of lactation
101	0·0466	1–2 weeks	101	0·0466	1–2 weeks
3	0·0499	3–4 weeks	3	0·0499	3–4 weeks
33	0·0563	2–4 months	15	0·057	2 months
17	0·0499	5–10 months	17	0·0558	3–4 months
			10	0·0521	5–7 months
			8	0·0468	7–10 months

These results show that the calcium content of the milk is lowest for the first two weeks of lactation, rising gradually to a maximum during the first four months, maintaining a fair level after the fourth month, but slowly decreasing. The same result is shown if our numbers are taken over rather shorter intervals of time, as shown on the right of Table I, indicating that the maximum lies between the end of the second and the beginning of the third month.

Among the earlier workers on this subject Hunnaeus [1909], Schloss [1911], Bahrdt and Edelstein [1910] all agree that the calcium in the milk decreases slowly with the course of lactation.

Bahrdt and Edelstein, while emphasising the high individual differences in the percentage of calcium in milk, concluded that their work showed this gradual decrease. Schloss alone mentions that he found the CaO less during the first week of lactation. The reason why the gradual increase from the first two weeks of lactation to a maximum at the end of the fourth month has not so far been emphasised is probably the fact that in many of the results recorded there are no samples taken until after the first month of lactation.

Table II gives a comparison of our results with those of other workers on milk. The figures of the latter have been averaged over the same periods.

Table II.

Authors			Holt		Telfer		De Buys and von Meysenburg [1924]		
Period of lactation	No. of samples	Mean value CaO	No. of samples	Mean value CaO	No. of samples	Mean value CaO	Period of lactation	No. of samples	Mean value CaO
First 2 weeks	101	0·0466	5	0·0444	5	0·0398	—	—	—
3–4 weeks	2	0·0499	6	0·0406	4	0·0407	—	—	—
					with rachitics 5	0·0422			
2–4 months (end)	33	0·0563	9	0·0483	9	0·0466	1–3 months	30	0·0435
					with rachitics 18	0·0477			
5–10 months (end)	17	0·0499	8 4–9 months	0·0453	5	0·0472	4–7 months	28	0·0392
					with rachitics 7	0·0474			
10–20 months	—	—	10	0·0388	12–13 months		8–12 months	12	0·0367
					1	0·048			
					with rachitics 4	0·044			
Total no. of samples	152	—	38	—	24 or 39	—	—	70	—

All these results show the same trend to a lower calcium value in the earlier months, gradually rising to about the end of the fourth month and then slowly sinking. Our values differ from those of the other workers in being slightly higher in value, the average value for the interval during 2–4 months being raised by several individual cases with a value 0·06 and there being no very low values. Some of the individual cases of the other workers are high, but their average is lower.

The explanation of the gradual rise in the percentage of calcium in the milk from the first week to the beginning of the third month and the gradual fall in the later months must be related to the physiological needs of the child.

During the first days after birth it must necessarily be trying to accommodate itself to its new environment and it is general that during the first week of life the weight of the child should decrease from that at birth. During this time it will need the minimum amount of calcium. As growth proceeds, according to the figures of Feldman [1927], the amount of milk taken per day increases from 300 cc. during the first week to 1000 cc. by the end of the sixth month. The increase in intake, however, is not uniform for each month. During the first month the intake rises from 300 cc. to 600 cc. per day, during the second month the average increase on the day's intake is 250 cc., during the third month the intake remains the same, and from the third to the sixth month the average rise is 50 cc. on the day's intake per month.

Table III shows the relationship between the volumes of milk and its calcium oxide content taken per day at the different periods of lactation.

Table III.

Period	1–2 weeks	3–4 weeks	2 months	3 months	4 months	5–7 months	8–10 months
Average CaO (g. per 100 cc.)	0·0466	0·0499	0·0570	0·056	0·055	0·0521	0·0468
Average volume of milk intake per day (cc.)	300	600	850	850	900	950 (5 months) 1000 (6 months)	—
Total intake of CaO per day (g.)	0·1398	0·2994	0·4845	0·476	0·495	0·495 (5 months) 0·521 (6 months)	—

This comparison shows that the gradual rise in percentage of CaO up to its maximum at the end of the second month coincides with the time when the baby is increasing its intake of milk by the largest average daily increase and presumably growing correspondingly fast. During the third month the intake of milk and the percentage of calcium remain practically constant. The gradual decrease in the percentage of CaO after the third month is counterbalanced by the continued increase in the intake of milk, so that the total intake of CaO during the first six months shows a gradually increasing value. The gradual decrease in the percentage of calcium will be balanced in the later months by the supplementary foods that the child will take about the age of six months.

PHOSPHORUS.

The estimations of phosphorus were made on the same samples of milk as were used for the calcium estimations. As no phosphorus estimations were originally made on early milk a separate series of experiments was carried out on the milk from 16 different women for this period. At present the phosphorus content of the milk of one individual has not been estimated over any consecutive length of time, but the cases so far examined on consecutive days or within a short period seem to indicate that as in the case of the calcium probably the individual differences in the phosphorus content are greater than those occurring in the samples of milk of the same individual. The same range of periods has been taken in averaging the percentage of phosphorus as in the case of calcium.

The results are shown on the left of Table IV.

Table IV.

No. of samples	Average P_2O_5 (g. per 100 cc. of milk)	Period of lactation	No. of samples	Average P_2O_5 (g. per 100 cc. of milk)	Period of lactation
14	0·0364	6th–14th day	14	0·0364	6th–14th day
4	0·0387	3–4 weeks	4	0·0387	3–4 weeks
37	0·0412	2–4 months	16	0·423	2 months
18	0·0410	5–10 months	21	0·0404	3–4 months
			9	0·0402	5–7 months
			9	0·0417	8–10 months

The percentages of P_2O_5 in the milk seem to run parallel with those of the calcium oxide. During the first two weeks of lactation the percentage of phosphoric acid is at its lowest. It then rises slowly up to the end of the fourth month when it remains practically constant up to the tenth month with a very slight tendency to decline. The variations in the percentage of phosphorus are not so marked as in the case of calcium.

When the above results are set out over shorter intervals of time, as shown on the right of Table IV, it is seen that the maximum percentage for phosphorus falls, as in the case of calcium, between the second and third months of lactation.

The percentage of phosphorus in milk, unlike that of the calcium which slowly decreases, remains almost constant after the fourth month. This may be due to the fact that, although the needs of the child for calcium and phosphorus for bone formation run parallel, it is probable that the demand for phosphorus for the growth of the soft tissues exceeds that for calcium.

The constancy shown by the percentage of calcium during the second and third month of lactation, when the intake of milk by the child remains constant, is not shown in the case of phosphorus. It is found that 0·0386 is the percentage of P_2O_5 for the third month as compared with that of 0·0423 for the second.

This work emphasises a point which Bahrdt and Edelstein [1910] also emphasised, viz. that it is only by comparing over long periods that any general tendency to decrease or increase can be observed.

Schloss [1911] showed, as we have done, a gradual increase in the percentage of phosphorus from the second week up to the third month of lactation, followed by a fall and rise but gradually decreasing to the eleventh month.

Telfer's [1924] results, on the other hand, agree in that they show a gradual rise to a maximum after the first two weeks and then a gradual decrease. His maximum, however, falls later, between the fifth and seventh months, and during the first two weeks his average is higher than at any other time. This may be due to the fact that it is only in the case of country women that the figures for early milk are recorded, and Telfer has pointed out that the percentage of phosphorus in the milk of these women is distinctly higher than that of town dwellers.

Schlossmann [1904] could establish no relationship between the percentage of phosphorus in milk and the duration of lactation, but, although his numbers vary, they seem to show that the percentage is gradually decreasing with the duration. Although there are exceptions, he thought that a high phosphorus content was associated with a high nitrogen content. So far we have no results to record on the percentage of phosphorus in milk on the first and second days after birth when the nitrogen percentage is very high.

Table V gives a comparison of our averages with those of Telfer and Schloss.

Table V.

	Authors		Telfer		
				P_2O_5 (g. per 100 cc.)	
Period of lactation	No. of samples	P_2O_5 (g. per 100 cc.)	No. of samples	All cases	Without samples from country women
5th–14th day inclusive	14	0·0364	5	0·048	—
3–4 weeks	4	0·0387	5	0·0384	(3) 0·0363
2–4 months	37	0·0412	17	0·0395	(13) 0·039
5–10 months	18	0·0410	6	0·0421 (6–8 months)	(5) 0·043
12–13 months	—	—	4	0·0377	(4) 0·0377

	Schloss						
	11–13th day	3–4 wks.	2 mths.	3 mths.	3½ mths.	4½ mths.	11 mths.
P_2O_5 (g. per 100 cc.)	0·0380	0·0393	0·0431	0·0501	0·0342	0·0415	0·0348

THE ASH AND ITS RELATIONSHIP TO THE CONTAINED CALCIUM AND PHOSPHORUS.

Our results on the amount of ash show that the ash is high in the early weeks of lactation, the average being 0·289 g. per 100 cc., while towards the end of lactation the average falls to 0·181 per 100 cc. Out of 77 samples during the first two weeks 43 showed an average of 0·3 g. or a little over, 19 of over 0·25 and the rest slightly lower. The above results are in accord with the work of Söldner [1896], who gave as his maximum for early milk 0·3 g. and his minimum 0·18.

The variations in the amount of ash during the course of lactation are given in Table VI.

Table VI.

Period of lactation	1–2 weeks	3–4 weeks	2–4 months	5–7 months	8–10 months
Average ash (g. per 100 cc. of milk)	0·289	0·249	0·243	0·236	0·181
No. of samples	77	4	37 2nd month 0·247 3rd month 0·2398	8	8

These figures show a rapid decrease during the first month of lactation, from the end of the first month to the seventh a slow decrease, and then again a rather rapid decrease during the last months, the figure during this last period usually lying between 0·1 and 0·2 g.

In considering the relationship of the calcium in the ash, the percentage of calcium rises from a mean of 15·5 during the first two weeks to 26·1 during the last three months, remaining fairly constant at 23 during the intervening period. These results are shown in Table VII.

Table VII.

Period of lactation	No. of samples	Average CaO (g. per 100 g. ash)	No. of samples	Average P_2O_5 (g. per 100 g. ash)
1–2 weeks	56	15·5	15	13·56
3–4 weeks	3	18·9	4	15·68
2 months	13	23·8	16	17·3
3 months	13	23·2	13	15·95
4 months	4	23·67	5	17·56
5–6 months	5	23·3	5	18·98
7–10 months	9	26·1	10	21·13

Schloss [1911] reports 11 % CaO in the ash of early milk, rising to 20 % in mature milk. The above higher percentages are probably due to the fact that the average calcium oxide in the samples of milk examined was higher than in those of Schloss.

In the case of phosphorus the percentage of P_2O_5 in the ash shows the same general rise from the first week to the tenth month of lactation, as is shown by the calcium oxide. The percentage, however, is lower throughout the course of lactation, rising from 13·56 in the first week to 21·13 during the last (seven to ten) months.

It is to be noticed here that the percentage falls between the second and third months and begins to rise again between the third and fourth months. This runs parallel with the phosphorus estimation, as it has already been shown that the percentage of phosphorus pentoxide in milk rises from the first week to the end of the second month, falls during the third month, rises again during the fourth month, after which a fair level is maintained with a very slow decline in the later months (Table VII).

RELATIONSHIP BETWEEN THE CALCIUM AND PHOSPHORUS.

Among the inorganic constituents of milk the calcium and phosphorus are the most important, and much attention has been focussed on these two elements in the diet in connection with the study of rickets.

It has been established that diets, insufficient in either calcium or phosphorus but adequate in every other respect, result in a retardation of the deposition of calcium phosphate in the bone and, where the deficiency of either element is too great, this cannot be counterbalanced by greatly increasing the antirachitic vitamins in the diet by the addition of cod-liver oil but only by the addition of the deficient calcium or phosphate. This has been established by the work of Sherman and his co-workers [1921, 1925, 1926] and confirmed by the work of McCollum and other workers on rickets.

McCollum *et al.* [1921] were the first to point out and emphasise the fact that the ratio between the calcium and phosphorus in the diet was of infinitely greater importance than the absolute amounts of the salts themselves. He showed that a deficiency of phosphorus in a diet, insufficiently supplied with "fat-soluble A," gave rise to rickets only when the calcium was present in an amount which gave a calcium-phosphorus ratio considerably above the optimum.

It is suggested that the importance of the ratio between the quantities of calcium and phosphorus in the diet is due to the fact that either excess of calcium or phosphorus, by precipitation of insoluble calcium phosphate in the intestine, would diminish the amount of phosphate absorbed.

Mellanby [1921] showed this to be the case with excessive amounts of phosphorus. The importance of this ratio has also been emphasised by Elliot, Crichton and Orr [1922] for pigs, and by Shohl, Bennett and Weed [1928] for rats.

In the present work this important relationship between the calcium and phosphorus has been studied in human milk. The latter is the natural food of the child during the first six months of life and therefore this ratio in milk should be the best for its nutrition and growth.

From the work already recorded on the variations in the percentages of calcium and phosphorus during the course of lactation a similar rise and fall in the ratio of these elements per 100 cc. of milk over the same period was to be expected. And since the variations in the percentage of P_2O_5 are similar to those of the CaO but are not so great, there should result less variation during the course of lactation in the ratio between these two elements than in the case of the elements alone.

It is found that this ratio, averaging over the same periods as before, does not show a large variation. The ratio rises a little from the first to the fourth month of lactation and then falls during the later months to a value less than that in the early weeks (Table VIII).

Table VIII.

Period of lactation	1–2 weeks	1 month	2–4 months	5–10 months
Average ratio CaO/P_2O_5	1·35	1·34	1·42	1·25 4–6 months 1·3 7 10 months 1·23
Ca/P	2·2	2·18	2·3	2·02
No. of samples	11	4	29	17

Although the average ratio over the whole course of lactation does not vary much, the individual variations are greater, varying from 0·81 to 2.

Out of the 61 samples investigated only 9 were between 1·8 and 2, of these 7 came in the period two to four months.

On examining the individual ratios it is found that 9 out of the 60 samples, *i.e.* 15 %, have a percentage of P_2O_5 greater than that of CaO, that in 7 samples the percentages are equal, and in all the other 45 cases the percentage of CaO is higher than that of the P_2O_5. In many of these cases the ratio is 1·1 or 1·2, but in some higher, the mean at the different periods lying between 1·3 and 1·4. When it has been possible to examine the milk of the same women at different stages of lactation the ratio between the percentage of CaO and P_2O_5, where high, certainly rapidly decreases and in the later months the P_2O_5 tends to become higher than the CaO.

Cases illustrating this are given in Table IX.

Table IX.

Case	Period of lactation	Ratio CaO/P_2O_5
H	6 weeks	1·88
	3 months	1·26
B	2 months	1·9
	3 months	1·3
G	4½ months	1·27
	5 months	0·94
	6 months	1·08
B	3 months	1·8
	6 months	0·91
Ha	9 months	1·01
	10 months	0·81

Although Schabad [1911] says it is not always possible during the first three months of life clinically to diagnose rickets, apparently all the children of the cases examined, with the exception of about two, were healthy children. Therefore our results show that, during the first six months of lactation, a ratio of CaO/P_2O_5 per 100 cc. of milk greater than 1 and approximating to 1·3 is probably the ratio most suitable for the child provided the vitamin factors are adequate.

That the factor can vary within quite wide limits is also evident and to be expected considering the extraordinary variability and adaptability of the human organism.

Since the ratio during the seventh to tenth months is decreasing (the only high ratio 1·7 being that of a mother with a rickety child, the high ratio being due to a low phosphorus content), it is possible that with milk after the tenth month, which we have not been able to obtain, the average ratio might become 1 or less than 1.

This wide variability of the calcium/phosphorus ratio is in accord with the work on rats which has been done in connection with rickets. Shohl, Bennett and Weed [1928] give the ratio Ca/P used by different workers [Boas, 1924, 1926; Medes, 1926; McCollum, 1922; Osborne and Mendel, 1913] as most suitable for their experimental diets and the ratio varies within the limits 1–2.

Telfer [1924] suggests that in milk a slight excess of phosphoric acid over the calcium equivalent necessary for bone formation is required for the growth of the soft tissue. In the case of his own results, however, out of 27 analyses of the milk of town dwellers, only two show a percentage of P_2O_5 greater than that of CaO. These cases Telfer considers abnormal, as in the analyses of the milk of 12 country women, 7 showed a higher percentage of P_2O_5 than of CaO, 2 an equal percentage, and only 3 a lower percentage.

The results of other workers, Schabad [1911], Schloss [1911], Söldner [1896], Burhaus and Smith [1923], confirm the variability of this ratio and show a higher percentage of calcium oxide in by far the greater number of cases.

Our results are certainly taken from samples of milk of women living in the poorer districts of London, but, considering the variations in the ratio, that the ratio is nearly always greater than one, that the children are healthy, and that many samples have been analysed, is there not some other physiological explanation for this ratio?

Sherman and Quin [1926], in their work on rats, showed that, during the suckling period, while there was a considerable increase of the soft tissues, where the phosphorus would be in organic combination, the largest gain in calcium and phosphorus was due to the deposition of these elements in the bones, and that, as tricalcium phosphate contains three atoms of calcium to two of phosphorus, the rapid gain of this substance must increase the ratio of the calcium to the phosphorus in the body as a whole. They found that at birth the atomic ratio of calcium to phosphorus in the body was 3 : 5 and that this had become 1 : 1 before the end of the suckling period. They showed that well within this period the percentage of calcium overtakes the percentage of phosphorus in the body.

If the same factors are operating during the suckling period of the child, the fact that the percentage of calcium oxide in the food, the mother's milk, is greater than that of the phosphoric acid has an explanation.

In support of this we have the fact that during the first six months of its life a baby doubles its weight at birth. The ratio CaO/P_2O_5 in tricalcium phosphate is about 1·2 and the mean of our ratios falls between 1·3 and 1·4. The gradual decrease in the ratio towards the end of lactation runs parallel with the decrease in the percentage of calcium oxide and phosphorus pentoxide during the later months, the decrease in the calcium being more marked than that of the phosphorus pentoxide.

There may be some differences in the milk of mothers living in the country and in towns as Telfer suggests, but the fact that nature produces a diet richer in calcium than phosphorus and the children bred on it are healthy must have some physiological explanation. The work of Sherman and Quin seems possibly to offer an explanation of the results recorded in this paper[1].

SUGAR.

Fifty-five samples of milk have been examined at different periods of lactation after the first fortnight (Table X). The method of estimation of lactose was that of Folin and Denis [1918].

There are two conclusions to be drawn from the results obtained.

(1) The percentage of sugar in early milk is at its lowest value.

(2) By the end of the first month the percentage has risen to an average which does not materially alter throughout the whole course of lactation up to at any rate the tenth month (no examination having been made of later milk).

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[1] (Note added 13th April, 1930.) Parsons (*Brit. Med. J.* 1927, ii, 780) states that "to obtain the maximum absorption of both calcium and phosphorus the diet should contain amounts of these elements sufficient in quantity and properly balanced. The percentage of calcium in the diet should be greater than the percentage of phosphorus."

Table X.

Period of lactation	1st–14th day	3–4 weeks	2–4 months	5–7 months	8–10 months
Average lactose (g. per 100 cc. milk)	5·845	7·08	7·099 2 months 7·18 3–4 months 6·97	6·93	7·09
Range of average	4·41–6·9	—	6·72–7·38 1 sample 8·4	5·83–7·35	6·12–7·77
No. of samples	115	2	29	12	12

To compare the percentage of lactose in early milk, that is up to the end of the first fortnight, with that found by other investigators, it has been found convenient to tabulate the results in days and these are given in Table XI.

No other observations have been recorded over the whole consecutive fourteen days for comparison with our results.

Although the numbers fluctuate a little there is a steady rise in the proportion of sugar during this period up to an average of 6·89 g., culminating in a value over 7 by the end of the first month.

Bracketed numbers refer to number of samples analysed.

Table XI. *Lactose* (*g. per 100 cc. milk*).

Days	Authors	Hammett [1917]	Bell	Camerer and Söldner [1896, 1898]	Söldner [1896]
1	4·74 (1)	—	—	—	—
2	5·56 (3)	—	—	4·782 (2)	—
3	5·33 (14)	5·43 (8)	—		
4	5·47 (11)	—	—	—	—
5	5·42 (16)	6·08 (8)	6·42 (88)	5·6 (2)	—
6	5·78 (13)	—	—		
7	6·17 (10)	6·21 (8)	—	—	—
8	6·34 (10)	—	—	—	
9	6·24 (9)	6·32 (8)	6·73 (88)	6·73 (1)	6·75 (2)
10	6·42 (9)	—	—	—	6·62 (2)
11	—	6·42 (7)	—	6·35 (1)	
12	6·12 (11)	—	—	—	—
13	6·69 (5)	—	—	—	—
14	6·89 (2)	—	—	—	—

Schlossmann [1900] recorded a value of 6·92 g. for the ninth to tenth day and Holt, Courtney and Fales [1915] gave an average of 6·5 for the first twelve days of lactation.

There is general agreement that during the first fortnight the average, although gradually rising, never rises to the figure 7 which is characteristic of mature milk.

From our results and those of Camerer and Söldner, it is probable that the percentage of sugar on the first day lies between 4 and 5, but too few samples have been examined on this day to establish this point.

The comparison of our numbers for the percentage of sugar in the later months with those of other workers is shown in Table XII.

Table XII.

Period of lactation	Authors	Schabad [1911]	Schlossmann [1902, 1904]		Holt, Courtney and Fales [1915]	Söldner [1896]	Camerer and Söldner [1896, 1898]
3–4 weeks	7·08 (2)	—	—	6·81 (65) from 11th day	7·81 12th–30th day 5·54 (1)	7·15 (4)	6·52 (15) 6·36 (4) 6·36 (7)
2 months	7·18 (14)	4·7 (2)	7·01 (30)	7·05 (56)	—	—	
3–4 months	6·97 (15)	4·72 (4)	7·35 (56)	6·87 (53)	7·98 (10)	7·42 (3)	6·81 (14) 6·66 (5)
5–7 months	6·93 (12)	4·36 (2)	7·11 (90)	6·89 (64)	8·15 (3)	—	6·87 (4)
8–10 months	7·09 (12)	5·48 (2)	—	7·33 (17)	7·66 (3)	7·28 (1)	6·78 (10) (5½–11 months)
10–20 months	—	—	—	—	7·41	—	—

Although our figures differ to a certain extent from those of the other workers, their general significance is the same. They all seem to indicate that there is an average percentage of sugar in milk which is well maintained throughout the course of lactation.

A different level of average percentages is to be expected where, as in all this work, the average is taken from a study of a large number of different individuals, as individual variations certainly exist. Schlossmann, for instance, finds in the case of some women a percentage of lactose as high as 10·75, while out of all our 55 samples only one was as high as 8. Considering also the variation in the methods of analysis of the sugar the differences in the averages are no greater than would be expected.

Hammett [1917] commented on the fact that the sugar estimations of the American workers were higher than those of others and certainly the results of Holt, Courtney and Fales [1915] are distinctly above those of the other workers recorded. Schabad's [1911] numbers are the only ones which are markedly different from all the others, the average being between 4 and 5 g. per 100 cc., the proportion associated by us with milk of the first few days of lactation. He also gets a very noticeable rise during the last few months. The small number of Schabad's samples may account for his figures.

In those cases in which we have been able to examine the milk of the same women at different stages in the lactation, there seems to be little variation in the percentage of the sugar except in two cases not included in the table.

This is evident from the cases quoted in Table XIII.

Table XIII.

Case	Month	Lactose (g. per 100 cc.)	Case	Month	Lactose (g. per 100 cc.)
A	5	6·8	E		7·35
	8	7·05			7·35
B	3	7·02	F		7·03
	6	7·00		7¾	7·18
C	9	7·31	G	1¾	6·97
	10	7·18		2½	7·34
D	1¼	6·94			
	2	6·90			

We have no explanation to offer of the two exceptions, nor has Schlossmann for similar variations that he records.

From the few cases at present examined, however, a pathological condition of the breast seems to influence the sugar percentage.

In one case, five days after birth, the percentage of sugar was 4·27 and in another case, fifteen days after birth, the percentage was only 3·97 and 3·65.

In all these cases the breast was very engorged and the percentage of sugar was found to be much below the average for the period of lactation.

Myers [1927] also gives a case of abscess of the breast, where at four and a half months the percentage of lactose is 2·16.

The low values for the sugar of early milk are of interest in comparison with the very general practice in the artificial feeding of young infants of the use of a highly sweetened food.

PROTEIN.

In the case of early milk it was shown that the highest percentage of protein occurred in the first three days after parturition and that the level fell steeply during the first week, to reach an approximate average of 1·4 % at the end of the thirteenth day. The present work on later milk shows that although after the first month the level is fairly constant for some months, yet a very slow continuous decrease is taking place from 1·4 at the end of the first fortnight to 0·9337 during the last month of lactation.

This gradual decrease is shown in Table XIV.

Table XIV.

Period of lactation	1st–3rd day	4th–7th day	8th–13th day	3–4 wks.	2 mths.	3 and 4 mths.	5 and 6 mths.	9–10 mths.
Average protein (g. per 100 cc. milk) (g. N × 6·37)	4·357	1·702	1·449	1·175	1·174	1·111	0·9855	0·9337
No. of samples	30	52	26	2	16	19	7	12
Range of percentages	8·47 - 1·19	2·586 1·18	1·984 0·98	— —	1·407 - 0·882	1·367 - 0·891	1·032 - 0·961	1·24 - 0·803
			1 only under 1	—	2 only under 1 and 2 only over 1·25	3 under 1 4 above 1·158	4 under 1	9 under 1 2, 1·035

There are several points of interest arising out of the above results.

(1) The percentage of protein during the first three days after parturition in milk rises in some cases to 6, 7, or 8, the highest value recorded being 8·4.

Other workers have noted high protein in early milk. Söldner [1896] records one between 5 and 6 % for the second day, Holt, Courtney and Fales [1915] 2·06 for the third day, and Hammett [1917] 5·79 for a third day, but no numbers are recorded as high as ours.

Among our estimations of the protein in milk during the first three days of lactation 6 are made on milk taken the first day after parturition, giving an average of 7·48 %, and 8 on the second day, giving an average of 4·58 %. This accounts for our high early values compared with those of other workers, for, with one or two exceptions, their earliest estimations are made on the third day of lactation.

It was thought possible, and suggested in our previous paper, that the high percentage of protein in the colostrum period might be connected with the small quantity of milk available at this period. Our earlier experiments [1927] support this explanation.

(2) It was also noticed in the cases examined that the initial protein values for the primiparae were in all cases much higher than those of the multiparae. The figures for the latter fell to a constant level much more rapidly than did those for the former. Since no distinctions of this kind have been recorded by other workers, comparison on this point is not possible.

In comparing values of the percentage of protein in milk it must be borne in mind that the several workers in this field have used different factors for converting the protein nitrogen obtained from the precipitated protein into protein itself. The factor used in this work is 6·37. Söldner, Schlossmann, Holt, Courtney and Fales use 6·25, Hammett and Bell 6·38.

In comparing the figures of these workers with ours, Söldner's numbers have been corrected to the 6·37 factor, but those of Schlossmann, Holt, Courtney and Fales are their recorded values.

(3) From the first day of lactation to the end of the first fortnight there is a rapid decrease in the average percentage of protein, the number falling from an average of 4·357 to 1·449.

(4) During the first and second months the level of the protein remains fairly constant. As lactation progresses, however, there is undoubtedly a gradual and continuous decrease to an average of about 0·9337 in the tenth month. This gradual decrease is also found by other observers.

(5) It is of interest that our range of numbers after the first fortnight are in accord with those of Söldner, the values in both series being considerably below those of Schlossmann and lower than those of Holt, Courtney and Fales. In the case of the latter workers the final average approaches ours, but not until much later in the course of lactation. Between five and six months our average is 0·9855 g. protein per 100 cc., while theirs becomes 0·972 after the tenth month.

A comparison of our results with those of other workers can be seen in the collected results in Table XV.

The average percentage of protein in mature human milk is recorded as 1·5 by Feldman [1927] and 1·6 by Sherman.

Our results suggest that after the first month of lactation a distinctly lower percentage of protein than 1·5–1·6 is present in milk, and that no single value can be taken as representative of the percentage over the whole course of ten months.

The fact that it has fallen to 0·934 by the end of the tenth month does not mean that the child is requiring less protein, as any slight lowering of the percentage of protein during the late months is counterbalanced by the larger volume of milk consumed.

Table XV. *Average protein (g. per 100 cc.).*

	1–3 days	4–7 days	8–13 days	3–4 wks.	2 mths.	3–4 mths.	5–6 mths.	7–10 mths.	11–20 mths.
Authors g. N × 6·37 per 100 cc.	4·35 (30)	1·702 (52)	1·449 (26)	1·175 (2)	1·174 (16)	1·111 (19)	0·9855 (7)	0·934 (12)	—
Camerer and Söldner [1896, 1898] g. N × 6·37 per 100 g.	2·05 (1) 1·624 (1)	1·77 (3) —	1·66 (10) —	1·22 (15) —	— —	1·019 (14) 3–4½ mths.	0·873 (10) 5½ and over	— —	— —
Schlossmann [1900] g. N × 6·25 per 100 g.	—	—	1·81 (5)	1·94 (41)	1·99 (30)	1·54 (60)	1·50 (60)	1·57 (30)	—
Holt, Courtney and Fales [1915] g. N × 6·25 per 100 cc.	—	2·206 (3)	2·19 (1)	1·17 (1)	1·05 (2)	1·141 (8)	1·13 (3)	1·27 (3)	0·972 (10)
Hammett [1917] g. N × 6·38 per 100 g.	3·52 (8) 3rd day	1·64 (16) 5 and 7 days	1·57 (15) 9 and 11 days	— —	— —	— —	— —	— —	— —
Bell [1928] g. N × 6·38 per 100 g.	—	2·0 (88) 5th day	1·73 (88) 9th day	1·37 (88)	1·30 (88) 5–6 wks. 1·21 (88) 6–8 wks.	—	—	—	—

Since human milk is the natural food of the child during its first six months of life these results should be of interest from the point of view of the preparation of artificial foods for infants.

SUMMARY.

1. The composition of human milk has been studied in the later stages of lactation and the calcium, phosphorus, ash, sugar and protein constituents compared with their proportions in early milk.

2. The percentages of calcium and phosphorus follow parallel curves throughout the course of lactation, though the variations in phosphorus are less than those in calcium. The percentage of both is less in the early weeks, rising to a maximum at the end of the second month, then slowly declining to the end of the tenth month. The relationship between the volume of milk assimilated and the percentage of calcium and phosphorus is noted.

3. The percentage of ash is shown to be highest during the first two weeks and then falls continually throughout lactation, the steepest fall being during the last month.

4. The percentage of both the calcium oxide and phosphorus pentoxide gradually rises in the ash to reach a maximum during the last months. That of the calcium oxide rises from 15·5 in the early weeks to 26·1 in the later months, while that of the phosphorus pentoxide rises from 13·56 to 21·13.

5. The ratio of the percentage of calcium oxide to that of phosphorus pentoxide in the milk has been studied at the different periods. It is shown to have a rise and fall following the corresponding rises and falls of the calcium

and phosphorus, but the variations are not great. The average rises from 1·35 to 1·42 and falls to 1·25 during the last months.

6. The fact that in the larger number of samples the percentage of the CaO is greater than that of the P_2O_5 is discussed and an explanation suggested.

7. The percentage value of the protein decreases rapidly from a very high value immediately after parturition to the end of the first month, and then gradually but continuously till it reaches an average of about 0·9337 by the end of the 10th month.

8. The figures for the average percentage of protein from the end of the first month till the tenth suggest that this is not a fixed value and that it has a lower average than 1·5 which is usually taken.

9. The percentage of sugar is at its lowest value in early milk. After the first month it varies little throughout lactation.

10. In those cases examined in which there was a pathological condition of the breast, the percentage of sugar is affected and the value lowered.

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XXXIX. FERMENTATION BY YEAST PREPARATIONS.

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THE striking difference between the fermentation of living yeast and that of the juice prepared from it is well known [Harden and Young, 1906]. A living yeast for example may ferment in an excess of sugar solution at a rate of 10–15 cc. CO_2 per 5 minutes for 2 g. of pressed yeast. The fermentation is unaffected by the addition of phosphate except in high concentrations, when a slight inhibition may occur. The yeast juice prepared from such a yeast ferments at 1/20–1/40 of this rate; the addition of phosphate to the juice produces temporarily a rate of the same order as that of the original yeast, which falls to the normal as the phosphate becomes esterified, but can be maintained indefinitely in a suitable concentration of arsenate [Harden and Young, 1911]. It seemed of interest to determine at what point in the preparation of yeast juice, or by what reagents or treatment, this modified fermentation was produced.

The preparation of yeast juice is usually carried out by grinding fresh pressed yeast with equal parts of silver sand and kieselguhr, and pressing out the ground mass after the addition of more kieselguhr in a hydraulic press. It is apparent that changes may occur either in the preliminary grinding, or by the retention of active material in the residue during pressing, or in both processes, and these were the possibilities first investigated.

Experiments on grinding yeast.

150 g. of fresh pressed brewery top yeast were mixed thoroughly with 150 g. of sand. A portion was removed to determine the original rate of fermentation, and the remaining mass ground in a mill, samples being removed at regular intervals for examination. The fermentations were carried out in the apparatus of Harden, Thompson and Young [1910] at a temperature of 30°. 4 g. of each sample (containing 2 g. yeast) together with 2 g. fructose and 20 cc. H_2O were incubated, the rate of fermentation and the response to phosphate being determined. Fig. 1 illustrates a typical experiment.

It will be seen that the fermentation rate (cc. CO_2 per 5 minutes) diminished approximately in proportion to the duration of grinding; after grinding for

1 hour (curve *c*) the rate had fallen from 13·5 cc. to 2·5 cc., and rose to 10 cc. on addition of phosphate, giving a typical phosphate curve. Microscopic examination of the ground mass at this stage showed that 70 % of the cells were now permeable to methylene blue, but no estimate was possible of the number which had been disintegrated by the grinding. Without addition of phosphate the rate of fermentation of the ground mass increased during 2 hours to double the initial value, corresponding then to the final rate after addition of phosphate; it is possible that this increase may be due to growth of the surviving intact yeast cells.

It appears from these results that the essential change in the mode of fermentation takes place during grinding, and that during pressing only secondary effects which may enhance this change can occur.

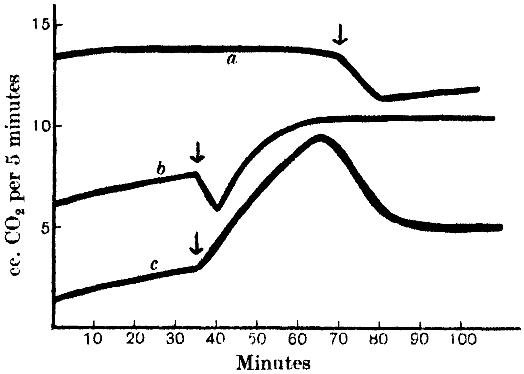

Fig. 1. Fermentation of yeast after grinding.
a, 2 g. yeast. *b*, 2 g. yeast ground 20 minutes.
c, 2 g. yeast ground 60 minutes.
↓ marks addition of 1·2 cc. *M* K_2HPO_4.

Experiments on grinding and pressing with various substances.

It was thought possible that the presence of the kieselguhr used as packing material during grinding and pressing, which could conceivably act as an adsorbent of active material, might account for the low basal rate of yeast juice. Preparations were therefore carried out in which other materials were substituted for the kieselguhr.

One of the difficulties encountered in the investigation of a series of yeast juices is the unaccountable manner in which juices prepared identically from different batches of fresh yeast from the same source vary both in their basal rate and their power to respond to phosphate. To provide a standard for comparison, it was therefore necessary on each occasion to prepare a juice in the normal way with kieselguhr.

Fresh pressed brewery top yeast was used throughout. The yeast was mixed with sand and the material to be tested, and ground to the usual consistency. After standing for 2 hours the ground mass was pressed out, and the volume and p_H of the juice were determined. Duplicate flasks were prepared containing fructose and a measured volume of juice and placed in a thermostat at 30°; the basal rate of fermentation and the phosphate rate were then determined. There was on occasion a noticeable difference in p_H between the experimental juice and the parallel kieselguhr juice, and in those cases a portion of the control juice was adjusted to the same p_H, without appreciable difference to the fermentation; the influence of the p_H during the fermentation may therefore be discounted, though it may be of importance in the preliminary pressing.

Table I.

Exp.	Packing material	Yield of juice cc./g. yeast	p_H	Rate cc. CO_2 per 5 min. for 25 cc. juice: Basal	Rate cc. CO_2 per 5 min. for 25 cc. juice: Max. phosphate
60	$CaCO_3$	100/500	6·0	8·0	51·0
61	$CaCO_3$	65/500	6·2	11·4	65·0
	Kieselguhr	65/500	6·0	7·5	74·0
62	$CaCO_3$	148/500	6·1	5·3	63·4
	Kieselguhr	160/500	5·4	1·9	29·0
72	$CaCO_3$	180/500	5·6	6·0	49·5
	Kieselguhr	154/500	5·4	1·5	26·0
66	Powdered glass	108/500	7·0	6·4	16·7
	Kieselguhr	170/500	5·6	2·4	16·0

Aluminium oxide, ferric oxide, barium carbonate and sulphate, and calcium phosphate gave juices which, though normal in yield and appearance, were inactive or of very poor activity. Magnesium carbonate gave a normal yield of juice with a basal rate lower than normal, which responded only slightly to phosphate; with aluminium hydroxide an extraordinarily poor yield was obtained, approximately 10 % of the usual volume; the juice however had a basal rate of fermentation twice that of the control but a phosphate rate of the same order as the control. The only substances used which had any beneficial effect on the fermentation rate were calcium carbonate and powdered glass. With calcium carbonate both the yield and the p_H of the juice obtained corresponded fairly closely with the control, but the basal fermentation rate was doubled or trebled. Table I shows some of the values obtained for the juices. Microscopic examination showed that both with the calcium carbonate and the kieselguhr, the number of cells in the ground mass permeable to methylene blue was the same, about 70 %.

To determine whether any active material could be adsorbed by kieselguhr, 50 cc. portions of the juices prepared with $CaCO_3$ were mixed with 5 g. kieselguhr, allowed to stand at room temperature for 10–30 minutes, and then filtered. On determination of the fermentation rate of the filtrate a slight diminution in the basal rate was observed; on one occasion the rate fell from 11·4 cc. CO_2 for 25 cc. to 9·5 cc. after treatment, and on another, from 5·3 cc. to 3·1 cc.

In the latter case the kieselguhr residue was suspended overnight in

20 cc. 0·1M K_2HPO_4, and the filtrate tested for the presence of hexosephosphatase by its hydrolysing action on potassium hexosediphosphate. It was found that 10 cc. filtrate, equivalent to 25 cc. original juice, liberated 0·4 mg. P in 5 minutes, which corresponds to a rate of 0·29 cc. CO_2, whereas the diminution in rate of the juice was 2·2 cc. Though the elution was far from complete, it demonstrated that active material was adsorbed on the kieselguhr. It was apparent, therefore, that though the main diminution in rate originated in the grinding of the yeast, the quality of the packing material might account for a decrease in rate up to at least 10 % of the total.

Effect of chemical reagents on fermentation by yeast.

The ideal reagent sought after in these experiments was one which, while killing the cell so that it was incapable of growth, would leave the fermentation rate unaffected. This was however not found. The action of some reagents has already been investigated. It was shown by Harden [1911] that the addition of toluene to fermenting yeast causes a rapid fall in the fermentation rate until a constant low rate is obtained, while simultaneously the yeast acquires the property of responding to phosphate. Kerr and Young [1926] showed that benzene, xylene, ether and ethyl butyrate acted in a manner similar to toluene, and that after treatment the yeast cells were incapable of growth; chloroform, ethyl acetate, amyl acetate and butyl alcohol completely stopped the fermentation.

Table II. *Fermentation of yeast in presence of various reagents.*

	Rates, cc. CO_2 per 5 min.			
Reagent	Original	After addition	Reaction to PO_4	% cells stained
Formaldehyde 2 %	44	4·0	–	15–20
Phenol 0·25 %	32	16·0	–	.
,, 0·5 %	30	1·0	–	.
Pyridine 2·2 %	22	7·0	–	.
Propyl alcohol 7·5 %	28	9·0	–	80
Quinone 2·5 %	26	15·0	–	.
,, 1 %	26	0	–	28
Toluene	32	1·5	+	100
Benzene	32	1·5	+	100
Carbon disulphide	28	2·0	+	100
Petroleum 12·5 %	27	3·5	+	100
Light petroleum 6·25 %	30	7·0	+ slow	75

A list of the reagents now investigated is given in Table II. The fermentations were carried out with fresh pressed brewery top yeast. A series of flasks was prepared containing a weighed amount of yeast, fructose and water and placed in a thermostat at 30°. When a steady rate of fermentation was obtained, usually after 10–15 minutes, the reagent to be tested was added to the flasks and the subsequent fermentation measured. After the rate had again become constant, the response of the fermenting mass to added phosphate was determined. A typical experiment is illustrated (Fig. 2).

(*a*) 8 g. pressed yeast + 3 g. fructose + 40 cc. H_2O.
(*b*) 8 g. ,, + 3 g. ,, + 40 cc. H_2O + 5 cc. carbon disulphide.
(*c*) 8 g. ,, + 3 g. ,, + 40 cc. H_2O + 4 cc. 4 % formaldehyde.

In Fig. 2 the arrow marks the addition of 2·4 cc. *M* K_2HPO_4 to *b* and *c*, the dotted portion (*b′*) of curve *b* representing the fermentation in absence of phosphate. In some cases a count was made microscopically of the proportion of cells staining with methylene blue after treatment with the reagent.

It will be seen that the fermentation was effected in one of two ways by the reagents tested. The first group, which includes phenol, pyridine and formaldehyde, reduced and with increasing concentration finally stopped the fermentation. No response to added phosphate occurred, indicating that the fermenting capacity was definitely lowered or destroyed; the cells, however, were not necessarily so injured that they stained with methylene blue. The second group, to which toluene, benzene and xylene were already known to belong, all reduced the fermentation roughly to the same degree, about 1/20 of the normal. The treated yeast then reacted with phosphate to give a definite phosphate rate, approximately 40–60 % that of the original yeast, and this rate could be maintained in the presence of arsenate. Moreover, on staining with methylene blue practically 100 % of the cells were permeable to the dye.

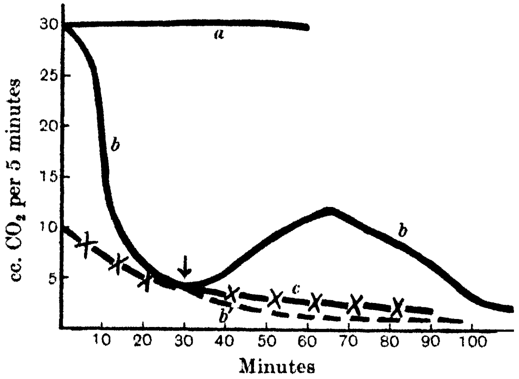

Fig. 2. Effect of reagents on fermentation by yeast.
a, Normal. *b*, In presence of carbon disulphide.
c, In presence of formaldehyde.
↓ marks addition of K_2HPO_4.

Assuming that in the living yeast fermentation takes place according to the equations of Harden and Young:

$$2C_6H_{12}O_6 + 2R_2HPO_4 \rightarrow 2CO_2 + 2C_2H_5OH + 2H_2O + C_6H_{10}O_4(PO_4R_2)_2,$$

$$C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O \rightarrow C_6H_{12}O_6 + 2R_2HPO_4,$$

or some similar equations, the enzymes must be so balanced as to produce the optimum concentration of phosphate. In the treated yeast, the limiting factor, as evidenced by the response to phosphate, is the hydrolysis rate of the hexosephosphates produced. The results quoted above lead to the conclusion that injury to the yeast cell, of which grinding is the extreme case and drying, and treatment with acetone, toluene etc. are intermediate gradations, results not in any significant diminution of fermenting capacity but in an inability to maintain a high rate and is confined to some special mechanism or organisation existing in living yeast which permits a more intense action of the hexosephosphatase.

The possibility therefore exists that by suitable treatment a substance could be extracted from yeast which would accelerate the action of hexosephosphatase and so increase the basal rate of yeast preparations, *e.g.* yeast juice or zymin, in a manner similar to arsenate.

Search for an accelerator of hexosephosphatase in yeast.

All attempts to prepare such an extract from living yeast, as by autolysis or plasmolysis, rubbing in a mortar etc. failed completely. Autolysis of a dried baker's (Dutch) yeast, which is a very active preparation fermenting at a rate 40 % that of living yeast, gave an extract accelerating the fermentation of zymin. 100 g. of dried yeast with 200 cc. H_2O were incubated for 16 hours at a temperature of 30° and the clear brown liquid separated by filtration. The accelerating power was measured in the following manner. Duplicate flasks were prepared containing 2 g. zymin, 2 g. fructose, 2·4 cc. *M* K_2HPO_4 and 10 cc. H_2O, and placed in a thermostat at 30° till a constant rate of fermentation was obtained. Preliminary experiment showed that this quantity of phosphate was sufficient to give the optimum concentration of phosphoric esters necessary for the maximum basal rate [see Boyland, 1929] and therefore no error would be incurred by the subsequent addition of extracts containing phosphate. A known volume of the autolysate was added to one flask and an equal volume of H_2O to a control flask, and the fermentation rate measured. In one experiment, the rate of fermentation was raised from 3·3 to 6·9 cc. CO_2 per 5 minutes, and in another from 3·85 to 8·05 cc. by the addition of 15 cc. autolysate. The acceleration was not produced if the autolysate was boiled previously to addition. Further examination of the autolysate showed that it contained an active hexosephosphatase. The hydrolysing power for potassium hexosediphosphate was measured under standard conditions at p_H 7·0, the inorganic phosphate liberated being estimated by a modification of Briggs's method. In one case 15 cc. autolysate, which produced an acceleration of 4·20 cc. CO_2 per 5 minutes in the rate of fermentation of zymin, hydrolysed 57·0 mg. P in 2 hours, corresponding to a rate of 1·8 cc. CO_2 per 5 minutes and thus accounting for 43 % of the acceleration. The figures obtained cannot fairly be compared, however, as the conditions existing during fermentation and hydrolysis are necessarily very different.

Attempts were made to separate the hexosephosphatase from any accelerating agent which might be present by means of precipitation with alcohol or saturated ammonium sulphate and by dialysis, but without success. From this point of attack, it remains impossible at the moment to decide whether the added hexosephosphatase alone is responsible for the acceleration, or whether it is accompanied by an accelerating system with which it is closely associated.

SUMMARY.

1. Investigation of the manner of preparation of yeast juice shows that at least 80 % of the diminution in the rate of fermentation which occurs may be ascribed to the process of grinding, during which the yeast acquires the power of responding to phosphate. A further loss is incurred during pressing by the use of kieselguhr, which adsorbs active material.

2. The effect of various reagents on the fermenting power of yeast is described.

3. The conclusion is drawn that the treatments described affect mainly the mechanism of hexosephosphatase action.

4. An account is given of the preparation, by autolysis of dried baker's yeast, of an extract containing hexosephosphatase and accelerating the fermentation rate of zymin.

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XL. PHOSPHORIC ESTERS IN ALCOHOLIC FERMENTATION.

II. PYROPHOSPHATE IN YEAST PREPARATIONS.

By ERIC BOYLAND (Beit Memorial Research Fellow).

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(*Received February 28th, 1930.*)

Lohmann [1928, 1] has isolated pyrophosphate from plants, the muscles of many animals and from living yeast. He has shown [1928, 2] that about one quarter of the total phosphorus present in both brewer's and baker's yeast occurs as pyrophosphate. More recently Lohmann [1929] has suggested that the pyrophosphate of muscle is combined with adenylic acid and may be concerned with ammonia production.

This idea is supported by the results obtained by Eggleton and Eggleton [1929] on the phosphorus compounds of muscle, and by the work of Embden, Hefter and Lehnartz [1930].

In studying the reaction of orthophosphate with zymin and yeast juice, Harden and Henley [1927] found that often only about 90 % of the theoretical amount of carbon dioxide was evolved. It is probable that the deficiency in carbon dioxide evolution is really due to depression of the basic fermentation to an unknown extent. The deficiency might, however, have been due to formation of pyrophosphate, but the present work shows that not to be the case.

Estimation of pyrophosphate.

Lohmann [1928, 1] and Eggleton and Eggleton [1929] have estimated the pyrophosphate of muscle as the phosphate liberated by hydrolysis for 7 minutes in N HCl at 100°. This gives reliable results with muscle, where there is no hexosediphosphate, but it cannot be applied to yeast preparations in which most of the phosphorus is usually present as the diphosphoric ester. In N HCl at 100° in 7 minutes hexosediphosphate loses about a third of its phosphorus as free phosphate. For this reason the old method of estimation of pyrophosphate could not be applied to yeast preparations.

In order to estimate pyrophosphate and hexosediphosphate it was necessary to find a method of separating these two compounds. Lohmann [1929] suggests that they can be separated as barium salts in slightly acid solution (p_H 4·5), but on trial this method was found to be not quite quantitative. Attempts were made to separate these two phosphates as the uranium, copper, chromium and iron salts, but largely on account of the complex pyrophosphate compounds, none of these methods gave a reliable separation. Finally the barium salts were used. While both barium ortho- and pyro-phosphate are insoluble in neutral solution, the salt of hexosediphosphate is soluble to the extent of 0·6 % in cold water.

The filtrate from the trichloroacetic acid precipitation was diluted until it contained less than 0·5 mg. phosphorus per cc. and was then treated with cold barium hydroxide solution till neutral to phenolphthalein and kept at 5° overnight. The organic phosphorus (hexosediphosphate and hexosemonophosphates) was estimated as the phosphorus left in solution as barium salt. The precipitate was removed by centrifuging and washed. It was then dissolved in dilute hydrochloric acid, the insoluble matter being removed by centrifuging and estimated as nucleic acid. The hydrochloric acid solution was again precipitated with barium hydroxide and the precipitate was assumed to be a mixture of ortho- and pyro-phosphate. By determination of the orthophosphate and total phosphorus present a figure was obtained for the pyrophosphate.

THE PHOSPHORUS COMPOUNDS OF LIVING YEAST.

The method was first used to estimate the phosphorus compounds of living yeast. Fresh brewer's yeast pressed to ½ ton per square inch was ground with trichloroacetic acid and an extract obtained by centrifuging. The results of analysis of the extract are shown in Table I along with similar figures given by Lohmann [1928, 2]. It should be noted that Lohmann heated his acid extract to 100° which might cause some hydrolysis and increase in the amount of orthophosphate present, and in his method of determination some of the hexosediphosphate would be estimated as pyrophosphate rather than organic phosphorus. Considering this fact the figures are in satisfactory agreement.

Table I. *Phosphorus compounds of yeast.*

	mg. P per g. yeast.	From Lohmann [1928, 2]	
	English brewer's yeast	Baker's yeast	Brewer's yeast
Total phosphorus	3·25	3·28	3·00
Orthophosphate	1·37	1·61	1·54
Pyrophosphate	0·68	0·79	0·69
Organic phosphorus	1·17	0·88	0·77
Hexosediphosphate	0·38	—	—
Hexosemonophosphate	0·72	—	—
Nucleic acid*	0·07	—	—

* The precipitate of nucleic acid was washed well with water so that some may have been lost.

THE REACTION OF PYROPHOSPHATE WITH YEAST PREPARATIONS.

The effect of adding pyrophosphate to a zymin fermentation was studied in the hope that it would throw light on the function of pyrophosphate in yeast. Pyrophosphate reacted in the same manner as orthophosphate but the delay in the reaction was a little longer.

The various amounts of phosphorus compounds present during the progress of the reaction were estimated by extension of the methods previously described [Boyland, 1929]. The results of such an experiment (Table II and Fig. 1) clearly indicate that the first change after the addition of pyrophosphate is the conversion of that salt into the orthophosphate, which then reacts in the usual way. Zymin and other preparations must therefore contain an active pyrophosphatase such as Kay [1929] has described as occurring in most mammalian tissues. The necessity for this conversion explains the slower liberation of carbon dioxide as compared with orthophosphate.

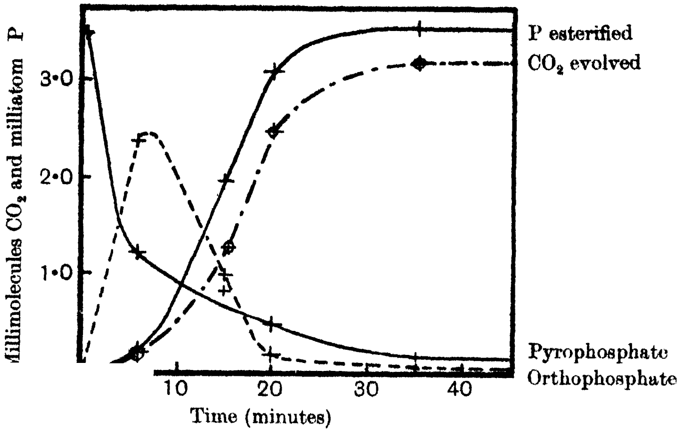

Fig. 1. Fermentation of fructose by zymin in presence of pyrophosphate.

Table II. *The reaction of potassium pyrophosphate with fermenting zymin.*

2 cc. *M* $K_2H_2P_2O_7$ added to 4 g. zymin in 25 cc. 10 % fructose solution.

Time after addition of pyrophosphate (min.)	Pyrophosphate present (milliatoms P)	Orthophosphate present (milliatoms P)	Organic phosphate formed (milliatoms P)	Carbon dioxide liberated (millimols)
½	3·5	0·18	—	—
6	1·2	2·36	0·13	0·10
15	0·8	0·98	1·95	1·24
20	0·5	0·13	3·11	2·50
35	0·1	0·02	3·57	3·20
45	0·1	0·02	3·57	3·20

PYROPHOSPHATE IN FERMENTING YEAST JUICE AND ZYMIN.

Yeast juice and zymin fermentations were carried out in the manner described in a previous paper [Boyland, 1929] with and without added phosphate and stopped in equilibrium by the addition of trichloroacetic acid. The amounts of orthophosphate, pyrophosphate and organic phosphate were then estimated in the trichloroacetic acid filtrate (Table III).

It will be seen that no appreciable amount of pyrophosphate is formed in the reaction and that it can be neglected in calculating the ratio of phosphorus esterified to carbon dioxide evolved.

The constant amount of pyrophosphate which occurs and remains unaffected by pyrophosphatase in the reaction is probably combined with adenylic acid in the way that Lohmann [1929] has suggested that it occurs in muscle.

Table III. *Pyrophosphate in yeast preparations.*

A. Exp. 146. 5 cc. 0·5 M Na_2HPO_4 added to 2 g. of acetone precipitate of yeast juice in 25 cc. 10 % fructose solution with acetaldehyde (0·1 %) present.

Phosphorus esterified	2·46 milliatoms
Carbon dioxide evolved	2·40 millimols in 40 min.
Pyrophosphate before addition	0·21 milliatoms
„ after reaction	0·24 „

B. Exp. 158. 5 cc. M K_2HPO_4 added to 4 g. zymin in 25 cc. 10 % fructose solution (with acetaldehyde).

Phosphorus esterified	5·0 milliatoms
Carbon dioxide evolved	4·9 millimols in 60 min.
Pyrophosphate before reaction	0·04 milliatoms P
„ after „	0·05 „
Nucleic acid before reaction	0·02 „
„ after „	0·03 „

THE ACETONE PRECIPITATE OF YEAST JUICE.

The deficiency in the amount of carbon dioxide liberated when phosphate is esterified by zymin fermentations is probably due to depression of the basic fermentation, owing to inhibition of the phosphatase, to an unknown extent. But when relatively small amounts of orthophosphate are added to fermentations the depression is small and under such conditions the carbon dioxide production approaches the theoretical amount.

Confirmation of the equivalence of esterification and gas formation was obtained with a preparation of yeast juice which had no basic fermentation of its own but reacted to phosphate, if acetaldehyde was present. Such a preparation containing no phosphatase could not have its basic fermentation depressed in the same way as the fermentation of zymin. Although almost 90 % of the phosphorus esterified was in the form of hexosemonophosphate, the carbon dioxide evolved was equivalent to the esterified phosphate. Results of typical experiments with such an enzyme preparation are given in Tables III A (for Na_2HPO_4) and IV. This indicates that the carbon dioxide evolved by esterification of phosphate is exactly equivalent to the phosphate no matter which ester is formed.

Table IV. *The result of the reaction of pyrophosphate with a fermenting preparation of the acetone precipitate of yeast juice.*

Exp. 146 B. 5 cc. 0·6 M pyrophosphate added to 2 g. acetone precipitate in 25 cc. 10 % fructose solution. Results expressed in total millimols.

1·68 millimols $Na_4P_2O_7$ (3·36 milliatoms P) were esterified and 3·12 millimols carbon dioxide were evolved in 60 min.

This dry preparation of yeast juice made by precipitation with acetone as described by Buchner, E. and H. and Hahn [1903] was treated with alcohol and ether and finally dried in a desiccator over sulphuric acid. For experimental use the dry powder was ground up with sugar and water; insoluble material was then removed by centrifuging. This removal of the insoluble material reduced the basic or hydrolysis rate of fermentation to a considerable extent, so that it would appear that hexosephosphatase is more readily removed by this treatment than are the other enzymes of zymase.

A preparation is thus obtained which has no hexosephosphatase but reacts with pyrophosphate in the presence of sugar and acetaldehyde. As pyrophosphate is hydrolysed before it reacts in this way, the preparation must contain a pyrophosphatase, and pyrophosphatase must therefore be a separate enzyme, distinct from hexosephosphatase.

Kay [1929] suggested that the pyrophosphatase action of mammalian tissues was probably due to orthophosphatase, although Lohmann [1928, 3] had shown that pyrophosphatase had a lower optimum p_{H} (7·0) than has orthophosphatase (p_{H} 9·0). This difference in optimum hydrogen ion concentration for the two enzymes supports the conclusion drawn above, that pyrophosphatase is a distinct enzyme.

SUMMARY.

1. A method for the separation of hexosediphosphate and pyrophosphate, depending on precipitation of barium pyrophosphate in dilute solution is described.

2. Pyrophosphate occurs in living yeast and forms about a quarter of the total phosphorus of yeast.

3. When pyrophosphate is added to fermenting zymin it is rapidly hydrolysed to orthophosphate which reacts in the usual way, hexosephosphates being formed and carbon dioxide liberated.

4. A preparation of the acetone precipitate of yeast juice did not ferment sugar (having no hexosephosphatase), but in the presence of acetaldehyde reacted with both ortho- and pyro-phosphate to liberate the theoretical amount of carbon dioxide and form 90 % hexosemonophosphate. This preparation must contain pyrophosphatase, which therefore would seem to be distinct from hexosephosphatase.

In conclusion I should like to thank Prof. A. Harden and Dr R. Robison for their help and criticism.

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XLI. NOTE ON THE DETERMINATION OF TOTAL CALCIUM IN BLOOD-SERUM.

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(*Received February 27th, 1930.*)

In a recent paper by Hendriks [1929], in which the application of the method of Trevan and Bainbridge [1926] to the determination of the total calcium of blood-serum was examined, certain discordant (low) results were attributed to a failure to adjust the heating conditions by which the calcium in the form of oxalate is converted to carbonate. Overheating leads to the formation of oxide which is difficultly soluble in the concentration of acid ($N/100$) used in the titration, while, on the other hand, underheating leaves some unchanged oxalate. In each case the final result will be low.

It seems both from the experience of Hendriks and that of the present author that the only weakness in an otherwise admirable method lies in the fact that the original authors did not lay down the aforementioned heating conditions in terms which would make them readily reproducible by other workers. However it was found that this defect could be overcome quite easily, and the method of Trevan and Bainbridge, as modified, has been used in this laboratory as a routine method practically from the time of its publication with entirely satisfactory results. It may therefore be of interest to record the modified procedure.

Following the original method of precipitation and washing of the oxalate precipitate, the remainder of the process was carried out in two stages.

(1) *Drying.* The special hard glass tube in its wire holder is kept inclined at a slight angle mouth downwards and laid on a pipe-clay triangle standing on a piece of plain iron-wire gauze stretched over a tripod, so that the tube is supported at a height of about 1½ inches above the level of the gauze. To provide a bed and a slope for the tube, a slight depression is filed in one side of the triangle, the mouth being over this depression while the conical end is supported over the opposite apex. Drying is begun by heating with a Bunsen burner placed under the gauze with the flame directed towards the open end of the tube, the heat then being taken gradually along to the closed end. Once the tube is dry the heating is increased to drive off the major portion of the ammonium oxalate.

(2) *Ignition.* To complete the process the tube is laid directly on the gauze itself, and the heating continued along its whole length and especially near the point where the calcium is packed. A period of about 5 minutes is allowed for this stage. The conversion to carbonate is accomplished well within this time, and with the gauze interposed no possibility exists of the process being carried too far, even though the Bunsen flame be used at its hottest intensity. It is both unnecessary and detrimental to heat in the naked flame.

For titration the acid is added (0·5 cc. of $N/50$ is preferred to 1 cc. of $N/100$ acid in that it provides for readier solution) without disturbing the solid particles, and with a thin glass rod slightly flattened at the tip, the carbonate while remaining at the bottom of the tube is ground as far as possible to a fine powder. In this form it goes almost instantly into solution when stirred into the acid.

The reliability of the above conditions may be illustrated by the following results.

(1) Solution containing 10 mg. Ca per 100 cc.

Mg. Ca per 100 cc. found: 9·93, 9·84, 10·01, 9·90, 10·02.

This series was obtained from a single sample by reprecipitating at the end of the first titration with ammonium oxalate, and repeating the whole process to the second and subsequent titrations.

(2) Normal blood-serum.

Mg. Ca per 100 cc. found: 10·23, 10·33.

Clark and Collip's method [1925]: 10·09.

The figures show only a slightly greater deviation from the mean than those quoted by the original authors, but such increased experimental error as does exist may be justly ascribed to the fact that the results recorded here have been obtained under essentially routine conditions, and without doubt could be still further improved with greater attention to the finer points of the technique.

SUMMARY.

Some improvements are suggested in the technique of the Trevan and Bainbridge method for the determination of the total calcium of serum. In particular a simple modification of the heating conditions is described which brings the oxalate-carbonate conversion under complete control.

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XLII. STUDIES ON GELATIN.

XI. DIAMINO-NITROGEN OF GELATIN.

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(*Received February 26th, 1930.*)

THE problem of the inherent stability of the protein molecule is one which has often been discussed, but upon which very little definite evidence is forthcoming.

When the colloid protein particle is envisaged as a complex amino-acid structure, there emerges the possibility that this structure may be subject to change. Changes in the surrounding conditions may produce changes in the internal structure, which in their turn would lead to alterations in the external behaviour of the molecule.

The fact that such external changes do occur may be instanced by the fall in viscosity, and ultimate loss of gelling power, of gelatin solutions on heating, but the occurrence of internal changes, whether related or not to external behaviour, is not so readily observed. This is to be ascribed largely to the lack of suitable analytical methods.

In recent years, however, Linderstrøm-Lang [1929] claims to have shown for caseinogen, and Schryver and his co-workers for gelatin, that there is evidence of an almost continuous variation in chemical composition, dependent upon conditions of treatment of the protein. An analogous series of changes has been shown to occur in the denaturation of ovalbumin by Mastin and Schryver [1926].

One of the analytical methods available is the precipitation of a portion of the nitrogen of the hydrolysed protein with phosphotungstic acid. This nitrogen is generally ascribed to the diamino-acids, but it will be shown that this involves something of an assumption, and it is desirable provisionally to consider this fraction simply as the nitrogen precipitated by phosphotungstic acid, without assigning to it any theoretical basis.

It was shown by Knaggs [1923] that if gelatin be allowed to stand in cold acid before hydrolysis, the amount of nitrogen which is precipitated by phosphotungstic acid in the hydrolysate increases. Increases varying from 4 to 10 % on a total of 20·4 % were obtained by treating the gelatin with cold HCl for 48 hours and afterwards throwing into boiling 20 % HCl.

Schryver and Thimann [1927] found that the soluble gelatin produced when the purified protein is flocculated from alkaline solutions does not show this effect, while the insoluble form—produced at the same time—does. In the same way, gelatin which has been brought permanently to the sol form by heating with water shows no rise in the nitrogen of the precipitate when it is similarly treated with cold acid before hydrolysis. The rise in the nitrogen precipitated by phosphotungstic acid, brought about by treatment with acid before hydrolysis. is therefore not constant but varies with the type of protein and with the conditions.

Thornley [1927] showed that the amount of increase varied with the strength of cold acid with which the gelatin was treated, and obtained a curve with a maximum rise of 5·8 %, corresponding to 2·5*N* cold HCl. In very strong acid a slight decrease took the place of an increase. Schryver and Buston [1927] traced the increase in this phosphotungstic precipitate to quantities of additional lysine; they found an increase of 1·6 % of ordinary lysine and 4·1 % of *dl*-lysine over the amounts present in gelatin hydrolysed in the normal way.

Daft [1929], however, published results which disagreed with the above and which apparently showed that no rise was produced by treatment of gelatin with cold acid before hydrolysis. In his experiments the gelatin was hydrolysed with and without pre-treatment with cold acid, and the nitrogen precipitated by phosphotungstic acid was shown to be constant. In the course of these experiments, however, the gelatin was heated with water (for a length of time which is not stated), before treatment with cold acid, and this may have seriously modified the results. It has, in fact, been observed by Knaggs and Schryver [1924] that boiling with water increases the non-amino-nitrogen of gelatin. Daft has therefore compared the value for the nonamino-nitrogen of the heated gelatin used in his experiments with that of the original gelatin before heating, and has shown that there is no great difference in the figures; he has thereupon assumed that his gelatin could not have been materially altered by this treatment. On the other hand, his value for the nonamino-nitrogen, 27 % as determined by the Van Slyke method, does not agree with the 12 % quoted by Knaggs and Schryver, and it is therefore possible that his gelatin had already been heated with water in some way before use, so as to increase the nonamino-nitrogen to a value which would remain unchanged on further heating. Now it has been shown [Thornley, 1927] that gelatin which has been boiled with water for 24 hours no longer gives a rise in phosphotungstic acid precipitate after standing in cold acid. Daft's gelatin may therefore have been boiled with water for a sufficient time to produce this effect. No information is given as to the source of his gelatin, or as to the methods adopted for its purification, so that it is impossible to determine how far this criticism affects his figures. Another point with reference to this work is discussed later (p. 366).

Whether this be the correct explanation or not of the disagreement between the results of Daft and those previously published, it was obviously desirable to reinvestigate the whole matter, for the existence of similar discrepancies has already been noted by other workers. Thus, Knaggs and Schryver [1924] found in one experiment that treating gelatin with cold *N* HCl produced no rise in the nitrogen precipitated by phosphotungstic acid, but only a rise in the nonamino-nitrogen. Thornley, on the other hand, obtained a considerable rise by treatment with cold *N* HCl. A careful examination of the method of analysis brought to light certain causes for variation in the amount of nitrogen precipitated by phosphotungstic acid.

(*a*) Different samples of phosphotungstic acid were found to produce slightly different amounts of precipitate; the crystalline acid of Kahlbaum yielded higher figures than the amorphous compound prepared in the laboratory both for gelatin and for pure arginine.

(*b*) Heating in the autoclave before precipitation, as recommended by Knaggs, was shown to cause a steady fall in the amount of nitrogen in the precipitate, varying with the time of autoclaving.

(*c*) The temperature at which the amide-nitrogen is determined has also a slight effect.

These factors are dealt with below.

None of the differences caused by these experimental variations was, however, great enough to account for the discrepancies observed, and attention was therefore directed to the concentration of nitrogen in the solution. The influence of this factor has long ago been noted [Thimann, 1926; Kernot and Knaggs, 1928], but early experiments had indicated that. if the concentration were kept within certain limits, constant values could be obtained. Those experiments were carried out, however, at relatively high concentrations, using the macro-method. The more convenient micro-method involves more dilute solutions of the amino-acids, and investigation of the effect of changes in the nitrogen concentration showed that under these conditions the amount of phosphotungstic acid precipitate varied almost continuously with the concentration of nitrogen. A single determination of this phosphotungstic precipitate therefore means nothing, and it is essential to plot the curve of "nitrogen concentration" against "percentage nitrogen precipitated" in order to obtain figures of any value. If this is done, curves are obtained which are characteristic of the protein used and are highly sensitive to differences in the treatment of the protein before hydrolysis. They therefore form a valuable means for comparison, and further afford definite evidence of the ready occurrence of intramolecular changes in gelatin under various treatments.

METHOD.

The micro-method adopted has already been described [Thimann, 1926], and the only modification introduced is in regard to the amide-nitrogen (see p. 360). The gelatin gel was warmed to 40°, made up to 15 cc. with water, and poured into 15 cc. boiling conc. HCl, so as not to interrupt the boiling. Hydrolysis was continued for 21 hours under a hang-in reflux condenser, the liquid rapidly cooled, 10 cc. samples were pipetted off and total nitrogen was determined on the remainder. The 10 cc. samples were evaporated *in vacuo* to remove acid, and diluted.

Determination of the amide-nitrogen.

2 cc. 10 % NaOH and 1 drop 1 % phenolphthalein were added, 1 : 1 H_2SO_4 was run in drop by drop till the solution was just acid, and 2 % NaOH added till just alkaline, as previously described. The liquid was transferred with washing to the Parnas and Wagner distillation apparatus and the ammonia distilled off in steam into $N/70$ H_2SO_4. Four minutes' distillation, followed by half a minute's steaming with the $N/70$ acid lowered from the nozzle were sufficient. The liquid was allowed to suck back into the trap and the apparatus washed out three times with vigorous shaking. The liquid was finally re-acidified with one drop H_2SO_4, evaporated down, and precipitated with phosphotungstic acid at 0°, as previously described.

Comparison was made with the aspiration method, both in regard to the amide-nitrogen and the phosphotungstic precipitate, in case the treatment with boiling alkali should have affected the latter. Gelatins of high amide content, and also gliadin, were used, to obtain better comparison (Table I).

Table I.

	Amide-N determined at 100° (4 min.).		Amide-N determined at 40° (90 min.).	
	Amide-N %	N in phosphotungstic ppt.* %	Amide-N %	N in phosphotungstic ppt.* %
Gelatin 1	3·20	14·98	2·58	15·80
Gelatin 2	4·39	14·90	3·70	15·24
Gliadin	26·75	—	26·30	—

All values represent the mean of two determinations.

* Low diamino-figures because dilute solutions used. See latter half of paper.

The boiling method therefore yields a higher value for the amide-nitrogen, lowering the N in the phosphotungstic acid precipitate by approximately the same amount. There is no reason, however, to believe that the lower figure is the more correct. If boiling with alkali drives off any nonamide-nitrogen, the amino-acids so decomposed should be arginine and cystine. Since gelatin contains no cystine, experiments were carried out with pure arginine. Distillation by boiling for 4 minutes, even with 40 % NaOH, however, yielded an amount of ammonia scarcely higher than the blank value. (The possibility of the partial scission of arginine into ornithine and urea, however, should not be overlooked (cf. following communication).) It is therefore safe to assume that no true diamino-nitrogen is distilled off under these conditions, and since the boiling method is quicker and provides closer agreement between duplicates it was used throughout.

Temperature of precipitation with phosphotungstic acid.

Daft states that precipitation in the cold yields a precipitate difficult to manipulate, and that the results were erratic, and recommends precipitation in the hot, followed by cooling. Comparison of the two methods, however, failed to yield any evidence for this statement, since the precipitates obtained in the two pairs of experiments carried out were identical in appearance and consistency, so far as could be ascertained, and also in nitrogen content (Table II).

Table II.

N	Precipitated hot %	Precipitated at 0° %
Amide	1·75	1·73
Phosphotungstic ppt.	20·49	20·58
Filtrate	78·57	76·57
Total	100·81	98·88

All values represent the mean of two determinations.

The discussion of Daft on the experimental procedure introduces no other fresh considerations, the effect of salts having been already pointed out [Thimann, 1926].

Effect of autoclaving before precipitation.

The rise in phosphotungstic acid-nitrogen produced by treating gelatin with acid before hydrolysis was ascribed by Knaggs [1923] to formation of a difficultly hydrolysable peptide. This peptide was said to be destroyed by heating under pressure at 120° with 5 % H_2SO_4 for 2–3 hours before precipitation. Since the bulk of the increase has been shown to be due to lysine and not a peptide, the effect of autoclaving seemed to call for explanation, particularly as it undoubtedly does lower the amount of nitrogen in the phosphotungstic acid precipitate. Check experiments were therefore carried out on a synthetic mixture of amino-acids which contained arginine, proline, and glycine. 10 cc. samples containing 5 % of H_2SO_4 were pipetted into short tubes, plugged with cotton wool, and autoclaved at 130° (Table III).

Table III.

Time of autoclaving (hrs.)	0		2·5		5·5	
	mg.	%	mg.	%	mg.	%
N in precipitate	1·22	20·58	1·16	19·68	1·04	17·63
N in filtrate	4·71	79·47	4·72	80·07	4·82	81·70
Total N	5·91	100·05	5·88	99·75	5·88	99·33

All values represent the mean of two determinations.

These experiments indicate that, quite apart from peptide-nitrogen, precipitable nitrogen of the true diamino-type is slowly destroyed by heating in the autoclave with 5 % acid. The use of this process is therefore not desirable, though the point might with value be subjected to a more complete investigation.

Effect of differences in the phosphotungstic acid used.

Three samples were investigated.

Sample A, prepared in the laboratory some time ago.

Sample B, freshly prepared. Both *A* and *B* had been prepared according to the standard method and thoroughly extracted with ether. They were amorphous white powders, whose solutions rapidly turned purple on standing. Sample A also yielded a slight deposit on long standing.

Sample C (Kahlbaum), was greenish and crystalline; its solutions became only faintly purple on standing.

The nitrogen content of the solutions was very low, being almost entirely due to the sulphuric acid used. For the 10 cc. of 2·5 % wash-liquid a blank of 0·12 cc. *N*/70 acid was obtained, while for the 3 cc. of 20 % precipitant the value was negligible.

10 cc. of a solution of pure arginine, containing 5 % H_2SO_4, were used for comparison. In duplicate experiments the precipitates were (*a*) centrifuged and then incinerated directly; (*b*) washed on the centrifuge four times with a total of 10 cc. of 2·5 % wash-liquid. The first gives a measure of the maximum precipitation possible, and the second the amount of precipitation which would be obtained under standard experimental conditions, since this is the usual procedure (Table IV).

Table IV. *Arginine solutions.*

Precipitates centrifuged without washing.

	Sample A			
	Fresh solution	3-months-old solution containing deposit	Sample B	Sample C
% N in ppt.	86·4	83·7	89·6	90·1
% N in filtrate	13·6	16·2	10·4	9·9
	Precipitates washed with 10 cc. wash-liquid.			
% N in ppt.	68·3	61·3	74·3	74·6
% N in filtrate	31·7	38·7	25·6	25·4

All values represent the mean of two determinations.

The use of stronger solutions of phosphotungstic acid, or larger volume of precipitant, gave in general lower values, though the differences were slight; the above amount, 3 cc. of 20 % solution, was therefore adhered to. It will be seen that the Kahlbaum acid and the freshly prepared laboratory material are identical within the ordinary limits of error, while the loss of efficiency in the old sample is considerable. Further comparison with gelatin hydrolysates showed a distinct balance in favour of the Kahlbaum acid, which was accordingly employed hereafter.

Table V. *Gelatin hydrolysate.*

N	Sample B %	Sample C %
Amide	2·06	2·07
Phosphotungstic ppt.	16·34	18·20
Filtrate	81·21	78·82
Total	99·61	99·09

It is not easy to assign reasons for the varying efficiency as precipitants of samples of phosphotungstic acid, but no doubt in such a complex molecule considerable differences in composition are possible, and even an ageing effect such as is observed above is not without precedent in inorganic materials.

Importance of the concentration of nitrogen.

It was stated above that earlier (unpublished) experiments showed that within certain limits the amount of nitrogen precipitated by phosphotungstic acid was independent of the concentration of nitrogen. These experiments were, however, carried out with Coignet's Gold Label gelatin, the use of which was afterwards discontinued on account of its uncertain origin. Further, the experiments were conducted under slightly different conditions and without the precautions shown since (and herein) to be of importance. The present experiments were carried out with a gelatin guaranteed to be prepared only from bone, and kindly supplied by Messrs Nelson, Dale and Co., of Warwick, to whom I wish to express my thanks.

As soon as precipitations had been carried out with this gelatin at different nitrogen concentrations, it became obvious that the principal source of disagreements lay therein. The curve, Fig. 1, shows the precipitation characteristic of this gelatin, compared with that of Coignet's Gold Label gelatin purified by flocculation, and it is clear that the former yields no flat range within the limits of concentration used (Table VI).

Table VI.

	N. D. and Co., Bone gelatin						Coignet gelatin (purified)			
Mg. N in 10 cc. solution	4·67	7·97	16·77	27·19	38·86	42·07	5·45	9·32	16·28	31·16
Amide-N (%)	1·60	2·18	1·89	1·65	2·01	1·74	2·19	1·77	1·58	1·59
"Diamino"-N (%)	15·89	18·72	23·34	24·53	22·48	20·54	16·61	19·66	22·38	22·75
Filtrate-N (%)	83·71	78·29	75·35	73·20	75·85	77·57	81·49	78·64	76·72	74·70
Total N (%)	101·20	99·19	100·58	99·38	100·34	99·85	100·29	100·07	100·68	99·04

EFFECT OF ACID ON GELATIN.

It was shown in the previous communication of this series [Schryver and Thimann, 1927] that if gelatin be dissolved in water, the solution made acid, and a potential difference applied across the solution, the gelatin is precipitated from solution as a gel instead of a white flocculum. Gelatin so treated has therefore been actually dissolved in acid and reprecipitated therefrom, and it is to be expected that the effect of acid applied in this way would be more complete than that produced by merely placing the dry solid in acid solutions for a corresponding length of time. In the latter case the effect will be modified by the relative ease with which the acid diffuses into the gelatin, *i.e.* probably by the water content of the gelatin. The effect may, of course, not be identical with that produced by the mere contact of the gelatin with acid; if, however a change is produced at all, it would be reasonably anticipated to occur in this case. Experiments were therefore carried out on the flocculation of gelatin from acid solutions.

The gel so produced was shown in the paper quoted above to possess properties differing from those of the flocculum obtained from neutral or alkaline solutions. Chemical differences were therefore to be expected.

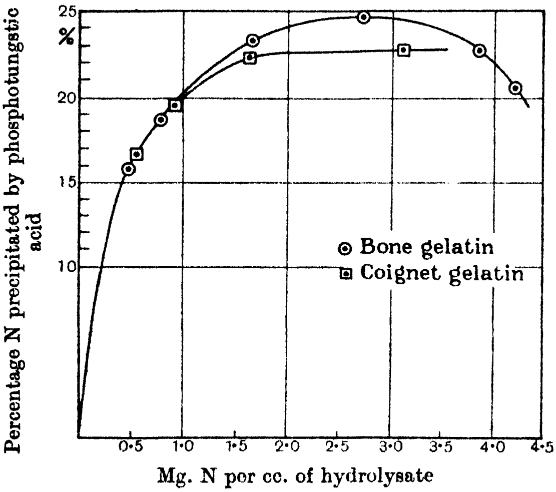

Fig. 1. Precipitation curves of gelatin.

Experimental.

The general procedure was as follows. 500 cc. of 1·5 % solution of the bone gelatin in water at 40° were brought to p_H 3 by addition of 60–70 cc. *N* HCl and electrolysed between collodion-muslin membranes. The outer vessels were supplied with slowly running freshly boiled distilled water, into which 1-inch square platinum foil electrodes dipped. A P.D. of 50 volts, *i.e.* about 2 volts per cm., was applied until the current began to fall from the original 5 to 2–3 milliamp. The P.D. was then slowly raised to 200 volts. After 10–12 days the current had fallen to 1 milliamp., and the p_H of the anode liquor had returned to 6–7. The stiff gel was removed, dissolved at 40° in 50–100 cc. water according to the dilution required, and cooled. For each hydrolysis the gel was warmed quickly to 40°, the required volume poured into 15 cc. boiling concentrated HCl, and water added if necessary to bring the volume of hydrolysate to 30 cc. The gelatin was preserved cold, under a layer of toluene. Samples having thus been removed for analysis, the remainder of the gel was redissolved in water at 40°, again acidified to p_H 3 and the flocculation repeated. On the second and subsequent occasions the resistance of the now salt-free gelatin solutions was considerably higher, and after passage of 1000 coulombs the final current fell below 0·2 milliamp. at 200 volts. In each case the gelatin was precipitated at the cathode membrane in the form of a stiff 10 % gel.

The Hausmann numbers of the gels were determined at several nitrogen concentrations. The amide-nitrogen varied irregularly between 1·5 and 2·0 %, although agreement between duplicates was close. The variation in the nitrogen precipitated by phosphotungstic acid[1] is shown by curve B in Fig. 2. Curves C, D and E give the variation for the same gelatin after the second, third and forth flocculation respectively from acid solution.

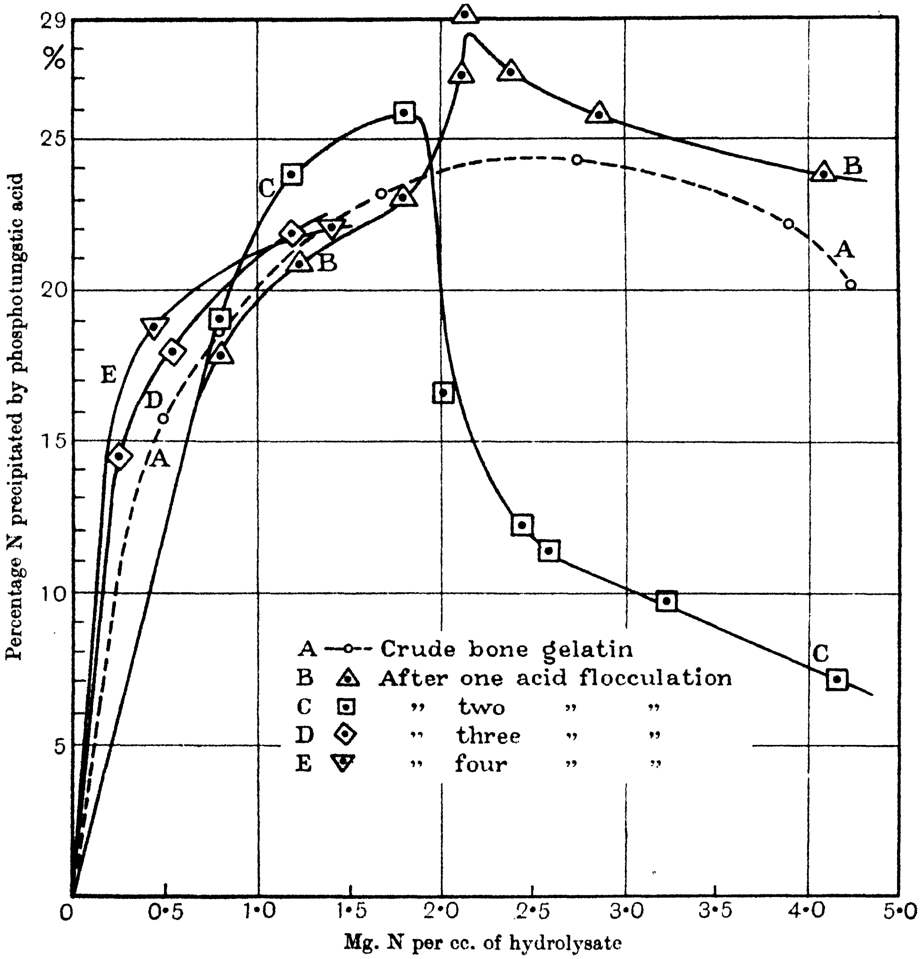

Fig. 2. Precipitation curves of acid-flocculated gelatin.

Some attempt to arrive at an interpretation of these curves has been made (see following communication), but at this point it will be sufficient to note that they indicate a continuous change in the amount of nitrogen precipitated, and a considerable difference from the original bone gelatin, the curve of which (A) is given for comparison.

When gelatin is flocculated from neutral or alkaline solutions it is obtained as a white hydrated flocculum, and it was thought to be of interest to compare this with the gels obtained above. A quantity of the crude bone gelatin was therefore dissolved to 1·5 % solution, brought to p_H 9 with KOH, and flocculated in the same way as the acid solutions but with the polarity reversed. Fig. 3 shows the curve (*F*) obtained with this preparation, compared with that given by the gelatin flocculated once from acid (B).

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[1] An explanation of the unusually low values shown on curve B is given in the following paper.

Comparison with other workers.

A word must be added here with regard to the concentrations of nitrogen used by other workers. The amount of gelatin used by Thornley [1927] coresponds to 5·6 mg. N per 10 cc., and it will be seen that although this falls on the steeply rising portion of the curve, yet even at this point quite definite differences should be experimentally observable between ordinary gelatin and that treated with acid before hydrolysis. The amount used by Daft, however, corresponds to 76·5 mg. per 10 cc., which lies altogether outside the scale of magnitudes possible for the micro-method. It should, however, be noted, that the slope of the curves of Fig. 3, if extrapolated, indicates for this concentration an approximate value for the nitrogen precipitated by phosphotungstic acid of 20 %: the value given in Daft's experiments averages 16·5 %, precipitated at 18°. This figure must be corrected to 0°, to correspond with those quoted here and this may be done by using his own figure of 2·5 % as the difference between the precipitates obtained at 0° and 18°. We thus obtain 19 % as the corresponding value at this concentration. It is therefore clear that Daft's figures lie close to the general curve, and, since these curves all appear to follow the same course at these high concentrations, no differences due to acid pretreatment in analyses carried out at these concentrations would be expected. This constitutes yet another

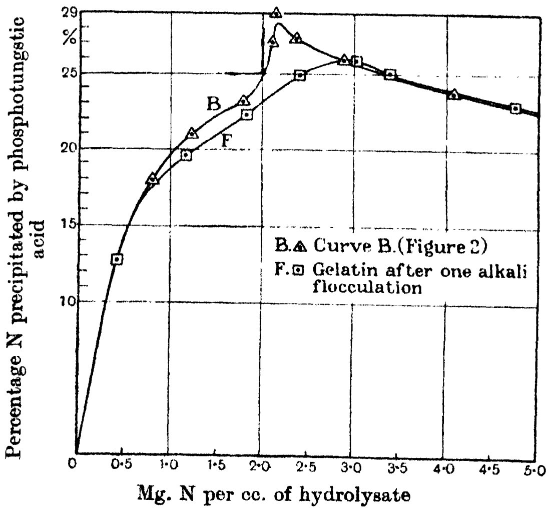

Fig. 3. Precipitated curves of acid- and alkali-flocculated gelatin.

reason for believing that this worker's results in no way disagree with those obtained by Schryver and others, previously quoted.

As was pointed out above, it has not been possible to repeat the analyses quoted by Daft, since no indication is given by him as to the nature and source of the gelatin used or its purification. Since the values obtained by him probably coincide with the curves as mentioned above, however, such repetition is scarcely necessary.

SUMMARY.

1. An investigation has been made of the reasons for the discrepancies between the results of different workers in regard to the amount of nitrogen precipitated from gelatin hydrolysates by phosphotungstic acid.

2. Differences in the precipitating power of different samples of phosphotungstic acid are shown to be responsible for subsidiary variations in this nitrogen.

3. Heating with 5 % sulphuric acid in the autoclave before precipitation destroys part of the precipitable diamino-nitrogen of arginine, and is therefore to be avoided as a step in the analysis of protein hydrolysates.

4. The amount of nitrogen precipitated by phosphotungstic acid varies almost continuously with the concentration of nitrogen in the solution, and in this fact lies the principal source of the discrepancies.

5. Curves showing the variation in the nitrogen precipitated with the concentration of nitrogen used must be substituted for single determinations in order to characterise the protein investigated.

6. Such curves clearly prove that treating gelatin with acid before hydrolysis profoundly affects the amount of nitrogen precipitated by phosphotungstic acid.

7. The protein molecule must, therefore, be regarded as an extremely labile structure.

8. A rapid method for the determination of the amide-nitrogen is described, and its effect on the subsequent precipitation with phosphotungstic acid is determined.

This work was done during the tenure of a Beit Memorial Fellowship, and part of it was carried out in collaboration with Dr A. B. P. Page, for whose assistance the author's best thanks are due. To the late Prof. Schryver, in whose laboratory the research was carried out, the author is most deeply indebted.

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XLIII. THE PRECIPITATION OF THE BASIC AMINO-ACIDS OF PROTEINS WITH PHOSPHOTUNGSTIC ACID.

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Although the fact has long been known that the diamino-acids are precipitated from solution by the addition of phosphotungstic acid, very little appears to be known as to the actual mechanism of the reaction. This is the more remarkable since the precipitation has been so widely used in biochemical research.

The effect of phosphotungstic acid on arginine was first studied by Gulewitsch [1899] who showed precipitation to be most complete in 5-7 % H_2SO_4, in which the solubility amounted to 70 mg. arginine per litre. He pointed out that "the composition of the arginine phosphotungstate can be different if the conditions of precipitation are different, especially if a mineral salt is present." Advocated as an analytical method for the estimation of the bases by Hausmann [1899] and modified by Osborne and Harris [1903] and others, the method has thus been in use for some 30 years. Osborne, Leavenworth and Brautlecht [1908] showed that the phosphotungstic acid precipitate agreed approximately with the amount of diamino-acids obtainable from the protein hydrolysate by other means. There were, however, one or two notable exceptions, and the application of later refinements in the technique of the isolation of amino-acids would undoubtedly reveal many more. Thus, Gortner and Sandstrøm [1925], working with a synthetic mixture of amino-acids, showed that precipitation was almost invariably too high, even after boiling the mixture, unless proline and tryptophan were both absent. Van Slyke's [1911] division of the nitrogen in the precipitate into arginine, histidine, cystine and lysine does not, of course, take account of the possible presence of other amino-acids in the precipitate; proline, for example, would be estimated as histidine.

Wechsler [1911] and Drummond [1918] carried out analyses of some of the phosphotungstates, whose general formula was shown to be A_3, $2H_3PO_4$, $24WO_3$, + H_2O in some cases, where A is the molecule of basic amino-acid. Drummond found the conditions of optimum precipitation for a dilute solution of histidine as being with 5 % H_2SO_4 (confirming Gulewitsch), and with

6–9 g. phosphotungstic acid per 100 cc. of resulting solution. Even under these conditions, however, 7 mg. N per 100 cc. remained unprecipitated, a solubility almost as high as that quoted in the same paper as the solubility in water, and four times as high as the minimum found by the present author (p. 374). It is evident that optimum precipitation conditions had not been attained. This may have been due to his use of an impure sample of histidine, or to failure to wash the precipitate with 2·5 % phosphotungstic acid solution.

Rôle of the basic amino-acids and proline.

There seems little doubt that the basic amino-acids are of the greatest importance in the structure of the protein molecule. Changes occurring in the latter appear to be reflected in, if not due to, changes in the content and proportion of these bases. Examples of such changes have been given by Schryver and Thimann [1927], Thimann [1926], Thornley [1927], Linderstrøm-Lang [1929], Knaggs [1929] and others.

Arginine has been considered by Kossel [1896] to act as the nucleus of the protein molecule, and a number of existing figures for the arginine content of proteins has been collected by Larmour [1928] to show the dependence of the amount of basic nitrogen on the amount of arginine in the molecule, and hence the governing importance of the latter. This is held to confirm Kossel's theory. Determinations of the amount of arginine are of necessity somewhat inaccurate, where protein hydrolysates are concerned, for two reasons. In the first place its precipitation by phosphotungstic acid is incomplete, as was pointed out by Gulewitsch [1899] and again by Plimmer and Rosedale [1925]. In the second place, in experimental procedure involving treatment with alkali, as in the determination of the amide-nitrogen of hydrolysates, the possibility of its decomposition has to be considered. Treatment with 50 % alkali, as in the Van Slyke procedure, yields ammonia, but only after 6 hours' boiling, so that this reaction need hardly be taken into account. Schulze and Winterstein [1901], however, showed that after 1 hour's hydrolysis with dilute alkali a 40 % conversion to ornithine and urea takes place. Although both these substances are precipitated by phosphotungstic acid, their precipitation in all probability takes place under different conditions from that of arginine, and the solubility of their phosphotungstates is certainly different, so that not only the arginine figures, but also the total phosphotungstic acid precipitate, would be affected. Since the hydrolysate is in fact treated with warm alkali for a short time the occurrence of this reaction is probable. This may perhaps act as a subsidiary cause for the variation of the ammonia-nitrogen also, though Henriques and Gjaldbäk [1910] attribute this mainly to deamination of some of the amino-acids, notably cystine.

Lysine is the seat of the bulk, at any rate, of the free amino-nitrogen of proteins, its ϵ-amino-group, which is free, being readily removed by nitrous acid, as was shown by Dunn and Lewis [1921]. It is, however, improbable that such deamination ever occurs in the ordinary way, since the product which would result, α-amino-ϵ-hydroxycaproic acid, has not been isolated from the products of protein hydrolysis. It may, of course, if present in small amount, have been overlooked, and its isolation in the future would be a matter of interest. Whether deamination occurs or not, the free nature of the ϵ-amino-group gives to lysine a unique importance in the protein molecule, and renders it probable that this amino-acid would be associated in some way with any intramolecular change that takes place. Its appearance in increased amount in gelatin which has been treated with acid [Schryver and Buston, 1927], supports this view. These workers obtained the bulk of the increase in the racemised or *dl*-form, but this, of course, may be merely a result of the method of isolation, which involved treatment with baryta and other possible racemising agents. Such racemisation is not without precedent, moreover, since Gulewitsch [1923] obtained racemic arginine among the products of gelatin hydrolysis, and ascribed the racemisation to the sulphuric acid used for hydrolysis.

Further evidence in support of the importance of this free NH_2-group is adduced by Hofmann and Gortner [1925] and Gortner [1927], who have shown that the combination between protein and acid dye takes place in stoichiometrical relation to the lysine-N + $\frac{1}{4}$ of the arginine-N, between certain p_H limits. Greenberg and Schmidt [1924] have in the same way compared the amount of combination with acid, calculated on this basis, with that actually found, for caseinogen, gelatin and gliadin, and obtained satisfactory agreement. Hitchcock [1923] showed that when gelatin was deaminised the reduction in combining capacity was exactly equivalent to the loss of amino-N. Much has been published on this point. Since the free amino-N, and therefore the lysine, of gelatin is thus responsible for its chemical combination with acids, it is to be expected that the results of acid treatment of gelatin would be closely connected with the lysine content. It is therefore not surprising to find, as will be shown herein, that a large part of the variations in the amount of nitrogen precipitated by phosphotungstic acid in gelatin which has been treated with acid may be ascribed to variations in the lysine content.

The consideration of cystine has been deferred to a later date, since the bulk of these researches have been concerned with gelatin, from which cystine is absent.

Proline is of importance because under such conditions it may be precipitated by phosphotungstic acid, as was pointed out by Gortner and Sandstrøm [1925]. Very few data relating to proline are to be found in the literature, and even those are chiefly derived from impure preparations, since it has only recently been obtained in a pure state by Kapfhamer and Eck [1927] and by Town [1928]. Its phosphotungstic acid precipitate is readily soluble in excess of phosphotungstic acid, and a permanent precipitate can only be obtained with concentrated solutions, or if the phosphotungstic acid is taken up by diamino-acids in the solution, so as to leave just sufficient to precipitate the proline. If the amount of diamino-acids were increased beyond this point, the total percentage precipitation obtained would decrease again, since there would not then be sufficient excess of phosphotungstic acid to precipitate these acids completely. For complete precipitation of the basic amino-acids an excess of the order of ten times their weight of phosphotungstic acid is required. Gulewitsch [1899] gives 12–14 times. This amount would suffice to redissolve the bulk, at any rate, of the proline, so that if the diamino-acids are completely precipitated, the proline is probably not, and *vice versa.*

The precipitation of any considerable percentage of proline would therefore take place only within certain limits of nitrogen concentration and at a certain ratio of proline to other amino-acids. Slight precipitation, however, probably takes place at most concentrations.

Variations in the precipitation of histidine with phosphotungstic acid do not seem to have been mentioned in the literature. No evidence of variation was obtained in the present work. Its phosphotungstate is more soluble in water than that of the other two bases.

It was shown in the previous communication [Thimann, 1930], that the amount of protein hydrolysate precipitated by phosphotungstic acid varies with the concentration of nitrogen in the solution. The experiments were therefore directed to determining the following points.

(*a*) The variation in the amount of single amino-acids precipitated by phosphotungstic acid with concentration of nitrogen.

(*b*) The constancy or otherwise of the solubility of the phosphotungstates.

(*c*) The behaviour of mixtures of amino-acids, and the interpretation of the curves given by gelatin hydrolysates.

EXPERIMENTAL.

Arginine was prepared *via* the flavianate by the method of Kossel and Staudt [1926], purified by way of the carbonate and obtained as a dry crystalline mass containing one molecule of water. Through the kindness of Drs Vickery and Leavenworth, samples of arginine, histidine, and lysine hydrochloride prepared by them [1928, 1, 2] were obtained. The arginine agreed closely with the above. Both the histidine and the lysine hydrochloride were finely crystalline specimens, containing 27·04 and 10·99 % N respectively; calculated 26·96 (free base) and 11·04 (hydrochloride + $4H_2O$). A sample of pure crystalline proline was very kindly supplied by Dr Town.

Solutions of weighed quantities of the amino-acids were made up, the nitrogen was estimated, and small volumes were measured with calibrated outflow pipettes. 0·3 cc. concentrated N-free sulphuric acid was added, the volume made up to 10 cc., and 3 cc. 20 % phosphotungstic acid in 5 % sulphuric acid solution were added. The tubes were allowed to stand in ice overnight, and the precipitates centrifuged, washed, redissolved, and incinerated as previously described for the determination of the Hausmann numbers

[Thimann, 1926]. The nitrogen of both precipitate and filtrate was determined on aliquot parts.

Two slight difficulties were encountered. Arginine phosphotungstate, when precipitated from dilute solutions, was fibrous and glairy, resembling egg-white, and therefore difficult to wash satisfactorily. It was eventually broken up with a fine glass rod of triangular section, which could also be used to cut the fibres. The rod was carefully preserved overnight and the adhering particles of fibre washed down into the centrifuge cup.

Lysine was not completely precipitated from the more concentrated solutions, and a secondary precipitate appeared in the filtrate when the wash-liquid was added. A further 1 cc. of 20 % phosphotungstic acid was therefore added to each and they were again centrifuged. Additional phosphotungstic acid added to the resulting filtrate produced no further precipitate. To keep the volume constant only 9 cc. wash-liquid were used. It is not possible that insufficient precipitant could have been added, since there was in these solutions a maximum of 39 mg. lysine to which 600 mg. phosphotungstic acid were added, *i.e.* a 15-fold excess. The probability is indicated that some type of soluble double salt was formed, and that this was decomposed by further phosphotungstic acid, thus:

$$6L + P = L_6P;\ L_6P + P = 2L_3P,$$

where L_3P is the normal insoluble salt (as given by analysis), and L_6P the soluble double salt.

This was the only amino-acid with which such a phenomenon was observed, and it is significant that the same phenomenon also occurs in the case of acid-treated gelatin. The formation of such a secondary precipitate, with incomplete initial precipitation, therefore points in such cases to a larger percentage of lysine (see below).

Table I. *Arginine* (*b*). (*Results typical of all experiments.*)

487·3 mg. in 50·01 cc.; *i.e.* 2·909 mg. N per cc.

cc. sol. taken	Total N mg.	Precipitate-N		Filtrate-N		Total recovery %
		%	mg.	%	mg.	
0·200	0·575	0·290	50·36	0·292	50·71	101·07
0·539	1·568	1·106	70·54	0·430	27·50	98·04
0·988	2·873	2·397	83·40	0·447	15·54	98·94
1·977	5·750	5·089	88·50	0·669	11·64	100·19
2·965	8·628	7·834	91·84	0·781	9·15	99·99

All values are the mean of two determinations.

Similar results were obtained with the other sample of arginine (*a*) (Vickery and Leavenworth). The curves (Fig. 1) show the precipitation in percentage at the different nitrogen concentrations for arginine (samples *a* and *b*), histidine, lysine and proline. It will be observed:

(1) that lysine shows the steepest rise, and is therefore the most insoluble;

(2) that precipitation is not complete in any of the cases, reaching 92 % for arginine, 91·6 % for histidine, and 98·4 % for lysine;

(3) that proline is only precipitated at very high concentrations, and then only in part;

(4) that the amount of nitrogen remaining in the filtrate, which should be constant if ordinary solubilities are involved, increases steadily with the concentration (see Table I).

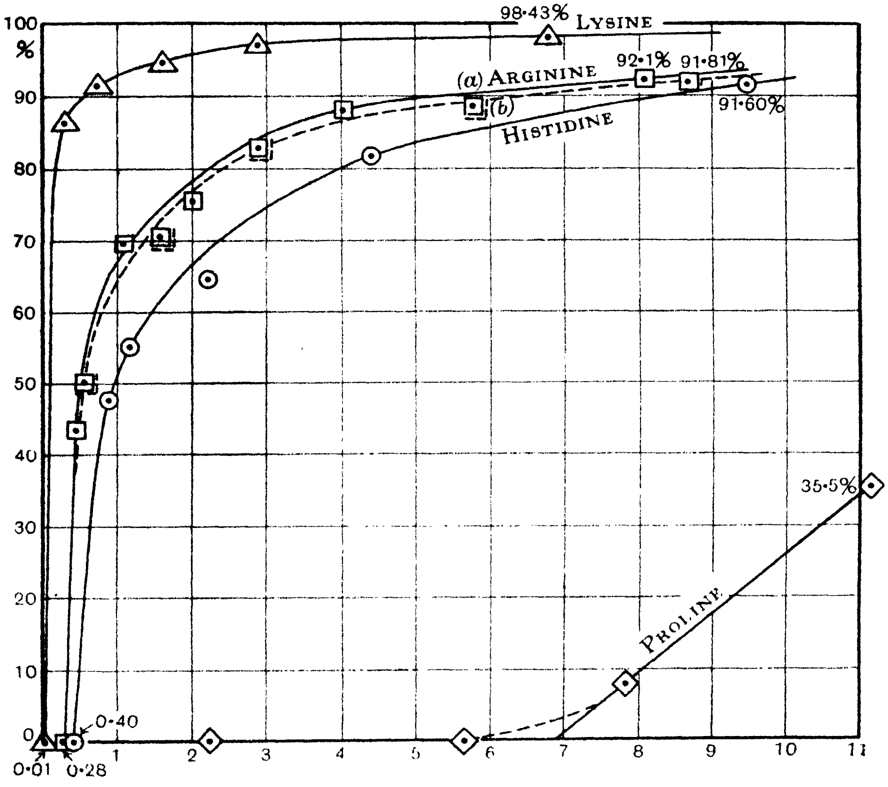

Fig. 1. Precipitation curves of basic amino-acids.

This last phenomenon may be due to two factors, the first of which is a true solubility in excess of amino-acid, and the second the presence of a quantity of non-precipitable impurity, such as a mono-amino-acid. This seemed improbable in view of the purity of the samples used, and its improbability was further confirmed in the following manner. Curves were plotted showing the amount of nitrogen in solution at different concentrations (see Fig. 2). Now the point at which the vertical axis is cut may be taken to represent the true minimum solubility of the phosphotungstate in 23 cc. of approximately 3·7 % phosphotungstic acid, 5 % sulphuric acid solution

(0·85 g. phosphotungstic acid having been added in all to 23 cc.). A line through this point parallel to the x-axis would represent a case of simple solubility.

In the case of arginine, however, the curve rises to an extent corresponding approximately with 0·45 mg. in 10 mg., so that 4·5 % of soluble impurity would have to be present to account for this slope. This quantity is altogether too large to escape detection and moreover would considerably alter the nitrogen figures, so that its presence, and hence this explanation, must be ruled out. It follows that the phosphotungstates of arginine and histidine, and to a much less extent that of lysine, are soluble in excess of the amino-acid. It may be that some kind of equilibrium between two salts of different solubility is established. In any case the precipitation is not simple.

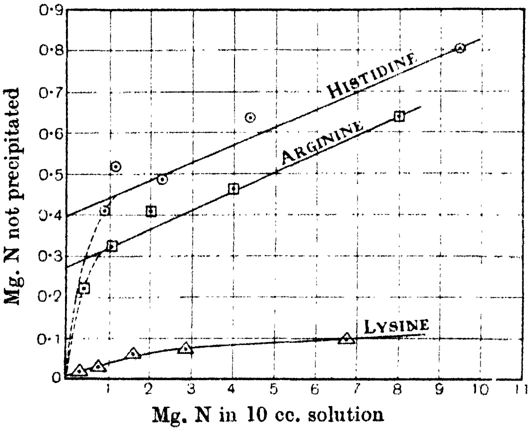

Fig. 2. Variation of the solubility of the phosphotungstates with nitrogen-concentration.

It seems at first sight a curious anomaly that lysine, which is known to give a soluble double salt in strong solutions, appears here as the least soluble of the three. This, however, is explained by the fact that in the two solutions of highest concentration, additional quantities of 1 cc. phosphotungstic acid solution were added in order to ensure complete precipitation. Apparently the formation of the soluble compound of lysine is on a somewhat different scale from those of the other bases, involving larger amounts when it occurs, but being more amenable to modified precipitation conditions.

The minimum solubilities as extrapolated from these curves are as follows (Table II):

Table II.

Minimum solubilities of phosphotungstates in 3·7 % phosphotungstic acid, 5 % H_2SO_4.

	Arginine (*a*)	Arginine (*b*)	Histidine	Lysine
mg. N per 23 cc. from curve	0·29	0·28	0·41	0·01
mg. amino-acid per 100 cc.	0·0072	0·0070	0·0115	0·0003
mg. N per 23 cc. given by Van Slyke	0·37	0·37	0·44	0·06

INTERPRETATION OF PRECIPITATION CURVES OF ACID-TREATED GELATIN.

Comparison of the curves for successive acid-flocculations of bone gelatin, given in the preceding communication [Thimann, 1930, p. 365] shows that they possess the following principal characteristics.

(1) A successive steepening of the rise from the origin, from the first to the fourth treatment.

(2) A rapid fall to very low values in the gelatin treated twice with acid (curve C).

(3) The occurrence of a peak in the curve for gelatin treated once with acid (curve B) or once with alkali (curve F), particularly the former.

It was shown above that the lysine curve is considerably steeper than that of the other amino-acids, so that the increase in steepness may be ascribed to increasing quantities of lysine. Confirmation of this is afforded by the steep fall. In all the points on the low portion of the curve, a considerable further precipitate separated when the wash-liquid was added to the filtrate, and 1 cc. additional phosphotungstic acid was therefore added and the precipitate again centrifuged. The addition of further phosphotungstic acid to the filtrate again caused a precipitate, and even after three additions of 1–2 cc. a precipitate was still produced on further addition of phosphotungstic acid to the centrifugate. This phenomenon is produced only by lysine, as shown above, and these facts therefore fall into line with the theory that relatively large quantities of lysine were present, and hence precipitation was incomplete throughout. Acid treatment therefore increases considerably the lysine content of these gelatin hydrolysates.

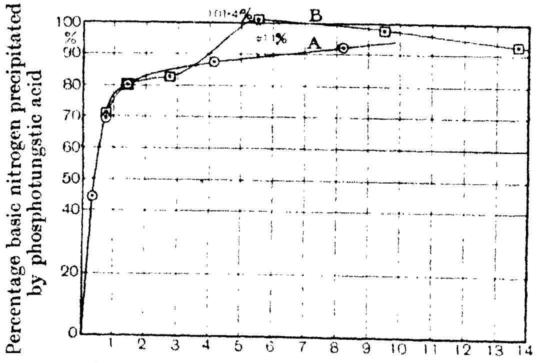

Fig. 3. Precipitation of amino-acid mixture with and without proline.

The peak. It was shown above that the precipitation of any considerable amount of proline takes place only at certain nitrogen concentrations and at certain proline-diamino-acid ratios. The suspicion that this maximum was due to proline was confirmed as follows. A mixture of the three bases was made up in approximately the same proportions as in gelatin, and its precipitation curve obtained (curve A, Fig. 3). To the same solution was added proline in corresponding proportions, and the precipitation curve again obtained (curve B, Fig. 3).

Mixture of bases.

		mg.	Proportions	
Solution A.	Arginine	35·4	5	in 100 cc. water.
	Lysine (hydrochloride)	286·4	4	
	Histidine	73·5	1	
Solution B.	Proline	237·5	3·5	in 30 cc. solution A.

A close similarity may be observed between curve B and that obtained for acid-flocculated gelatin (curve B, Fig. 2, of the preceding communication [Thimann, 1930, p. 365]). It is clear from these curves that proline is precipitated at certain concentrations only, and then only to the extent of about 11 %, but it is also abundantly clear that the peak of the gelatin curves is due to proline and can be imitated by addition of proline to the bases. The acid treatment of gelatin therefore increases the proline and lysine content.

All these curves show a general falling off at high concentrations, due to the slight solubility of the phosphotungstates in excess of amino-acid.

The phosphotungstic acid precipitation curves are therefore not merely useful as indicators of intramolecular lability, but are also amenable to interpretation and can yield valuable knowledge of the amino-acids present.

CRYSTALLISATION OF THE PHOSPHOTUNGSTATES.

During the course of this work opportunity was taken to crystallise the phosphotungstates in pure condition. Excess of phosphotungstic acid was not used in their preparation, in order to eliminate the possibility of the presence of the free acid.

Phosphotungstic acid itself crystallises well in large rhombohedral prisms[1] which quickly dehydrate and become opaque and friable on drying. The double refraction shown is very low. The angle between the well-developed rhombohedron and the basal plane is about 58°, giving an axial ratio of 1·6. The small crystals are shown in Plate I, Fig. 1, and a well-developed pyramid in Fig. 2.

Arginine phosphotungstate crystallises from water in 4- or 6-sided tablets, invariably twinned, of the monoclinic or triclinic system. They were erroneously described by Drummond [1918] as rhombohedral. The angle of extinction makes 27° with the composition plane. The refractive index is greater than 1·5 (Plate I, Fig. 3).

Histidine phosphotungstate may be obtained from water in the same form as arginine, but with greater difficulty and in smaller crystals. From acetone-water it separates in clustered prisms, also probably monoclinic (Plate I, Fig. 4).

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[1] For assistance with the crystallography I am greatly indebted to Mr M. H. H. Hay, of the Natural History Museum, South Kensington.

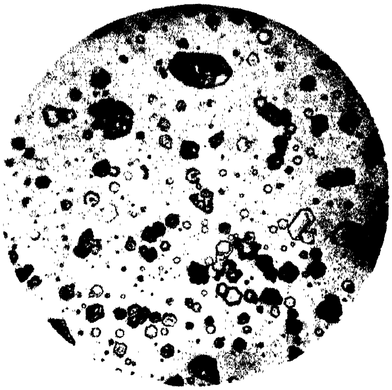

Fig. 1. Phosphotungstic acid.

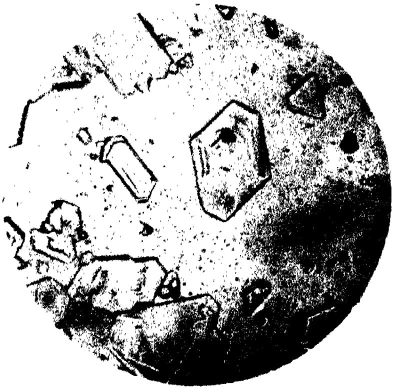

Fig. 2. Phosphotungstic acid.

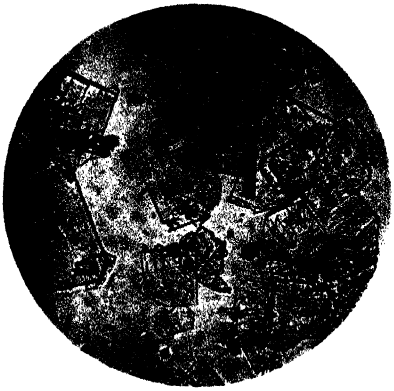

Fig. 3. Arginine phosphotungstate.

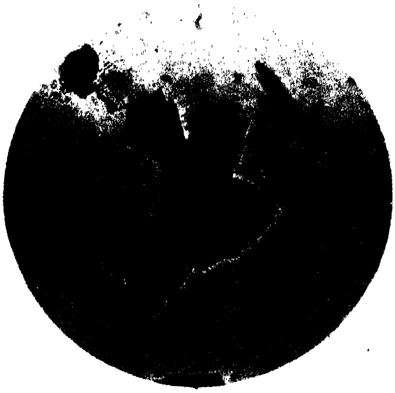

Fig. 4. Histidine phosphotungstate (prismatic form).

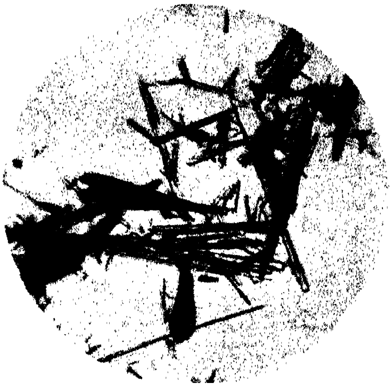

Fig. 5. Lysine phosphotungstate.

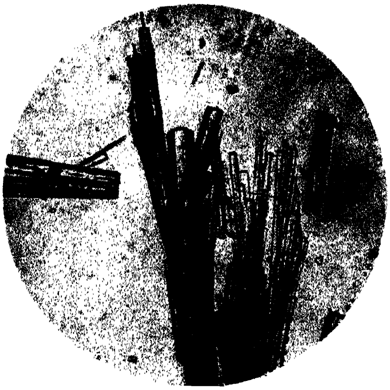

Fig. 6. Proline phosphotungstate.

Lysine phosphotungstate crystallises readily from water to which a few drops of acetone have been added in tiny clusters of fine orthorhombic needles, resembling wheatsheaves in the typical form. By slow evaporation needles 2–3 mm. long may be obtained (Plate I, Fig. 5).

Proline phosphotungstate separates from water to which a few drops of acetone have been added, in orthorhombic needles of large size, clustered together (Plate I, Fig. 6). The double refraction is high—about 0·04—and the refractive index greater than 1·5. By slow evaporation it can be obtained from water in small rhombs.

SUMMARY.

1. The rôle of the basic amino-acids and proline in the protein molecule is discussed, and the probable importance of lysine in intramolecular change is emphasised.

2. The precipitation of the individual amino-acids by phosphotungstic acid is determined at various nitrogen concentrations. It is not a simple phenomenon, the solubility of the phosphotungstates increasing with nitrogen concentration. In the case of lysine there is evidence for the formation of a soluble double salt. Proline is precipitated only at high nitrogen concentrations.

3. The minimum solubilities of the basic amino-acids in the final precipitating liquid are determined and found to be somewhat less than the solubility corrections given by Van Slyke. The actual correction to be employed in any experiment, however, will be considerably higher and will vary with the nitrogen content of the solution.

4. The precipitation curves of the individual amino-acids throw considerable light on the curves obtained with gelatin hydrolysates. In this case they show that the acid flocculation of gelatin produces an increased amount of proline and lysine.

5. The crystalline form of the phosphotungstates is described.

The author's best thanks are due to Drs Vickery and Leavenworth of New Haven, U.S.A., and to Dr Town, of St Bartholomew's Hospital, for the gift of amino-acids.

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XLIV. INVESTIGATIONS ON THE HYDROLYSIS PRODUCTS OF FISH MUSCLE.

I. THE DIAMINO-NITROGEN OF COD MUSCLE.

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AND NITA ELISE SPEER.

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(*Received February 28th, 1930.*)

In determining the diamino-nitrogen in the products of hydrolysis of proteins, Kernot and Knaggs [1928] showed that the results depend on several factors and, amongst them, on the temperature of precipitation and the temperature of the solution whilst the precipitate is settling.

Their results were confirmed by Daft [1929] who suggested that, in order to avoid discrepancies, a rigidly defined technique should be followed. Rosedale more recently [1929]. in determining the diamino-content of fish muscle, came to the conclusion that results are not so much influenced by temperature as by the incompleteness of the hydrolysis, since in this case material which cannot be considered as consisting of diamino-acids is precipitated by phosphotungstic acid.

There is, however, no experimental evidence as to products of incomplete hydrolysis being precipitated with the diamino-fraction, but, as pointed out by Kernot and Knaggs [1928], proline, tryptophan, phenylalanine and hydroxyaminobutyric acid have been found in this fraction.

The object of the present communication has been to find out how far the completeness of the hydrolysis could influence the results. If the hydrolysis is incomplete peptides may be found in the basic fraction but it is doubtful whether they could resist the prolonged action of a 20 % solution of sulphuric acid. This, however, would not be the case with some ketopiperazides which are so highly resistant to the action of acids and alkali that the question has arisen whether such products could be considered as primary constituents of the protein molecule. A preliminary attempt has been made to separate some of these products in order to find out whether they would be precipitated by phosphotungstic acid.

EXPERIMENTAL.

Cod muscle, from recently caught fish, was the material used for these experiments. The tissue was freed, as far as possible, from adjacent tissues and then dropped into boiling water, the boiling being continued for one hour. The coagulated muscular tissue was strained on a cheese cloth washed with distilled water and suspended in chloroform water. After 24 hours the chloroform water was changed and the treatment repeated 5 or 6 times. It was then again strained, digested with 95 % alcohol and then twice extracted with absolute alcohol. After a further extraction with ether it was air-dried.

Estimation of the diamino-nitrogen.

Four portions of 25 g. each of the dried muscle were hydrolysed with 600 cc. of a 20 % solution of sulphuric acid for 12, 24, 48 and 96 hours respectively. The hydrolysate was diluted with water and barium hydroxide added until it was only very slightly acid to Congo red. The barium sulphate precipitate was removed by filtration and the precipitate digested four times with boiling water. These washings were added to the hydrolysate, and the combined filtrates, to which a small excess of barium carbonate had been added, were evaporated to dryness under reduced pressure. The residue was taken up with water, filtered, made up to a known volume and the nitrogen estimated by the Kjeldahl method.

Aliquot parts of these solutions were taken and made up to 100 cc. so that each contained 80 to 85 mg. of nitrogen.

The diamino-nitrogen was precipitated with phosphotungstic acid according to the method of Osborne and Harris [1903], the conditions of temperature being as follows.

(*a*) The hydrolysate and the phosphotungstic acid solution were separately heated to 100°, and after addition of the latter to the hydrolysate the solution was allowed to cool and stand in an incubator at 37° for 48 hours.

(*b*) The hydrolysate and the phosphotungstic acid were separately heated to 37°, and after precipitation kept in an incubator at 37° for 48 hours.

(*c*) The hydrolysate and the phosphotungstic acid were separately cooled to 0°, and after precipitation were allowed to stand at the same temperature for 48 hours.

The experiments were carried out in duplicate, and the average results are given in Table I.

Table I.

Time of hydrolysis in hours	% of N precipitated by phosphotungstic acid		
	(*a*)	(*b*)	(*c*)
12	36·84	39·47	43·65
24	30·67	36·16	40·11
48	25·64	31·31	37·28
96	23·85	30·40	35·83

These results show that the percentage of diamino-nitrogen precipitated by phosphotungstic acid depends on the conditions of precipitation, and not only on the completeness of the hydrolysis.

Rosedale states that in the case of fresh fishes hydrolysis appears to be completed between 40 hours (carp) and 80 hours (tunny). We have found that the hydrolysis of the muscular tissue of fish may be considered as complete after 24 hours, as then the percentage of total amino-nitrogen reaches its maximum. If the time of hydrolysis is prolonged this total diminishes while the percentage of nonamino-nitrogen increases, possibly owing to the formation of ring compounds.

In the following section we give a preliminary account of experiments carried out with the object of obtaining, if possible, some evidence on this subject.

Separation of some products of partial hydrolysis.

Abderhalden [1923] and his co-workers have, by means of solvents, isolated several products of the partial hydrolysis of proteins. The method consisted in evaporating the hydrolysate to dryness under reduced pressure, mixing the residue with sand and extracting it with solvents. By this method it is possible to use a great variety of solvents, but as all ketopiperazines are soluble in many solvents which are not miscible with water it was thought that a modification of the method suggested by Dakin [1912, 1920] could be used.

By extracting the hydrolysates from proteins with butyl alcohol, under the conditions described by Dakin, the proline fraction could never be obtained free from diketopiperazines, and in order to avoid this, Osborne, Leavenworth and Nolan [1924] suggested that the extraction should be carried out at room temperature. Even in these circumstances, however, ketopiperazines were found to be constantly present in the fraction.

In the following experiments, besides butyl alcohol, ethyl acetate and chloroform were used. The extractions were carried out by shaking the hydrolysates in a separating funnel with an excess of the solvent; they were repeated until a small portion of the solvent on evaporation left no residue. As in the previous section the experiments were carried out in duplicate and the figures given represent averages.

No attempt has been made, at this stage, to identify the different products obtained.

Ethyl acetate extract. The following percentages of the total nitrogen in the hydrolysate were extracted by this solvent.

Time of hydrolysis in hours	12	24	48	96
% N	2·02	0·25	0·16	0·10

This substance has all the characters of a diketopiperazine as it is practically insoluble in water, sparingly soluble in cold ethyl alcohol, but easily in hot ethyl alcohol, from which it crystallises on cooling in fine needles melting at 175° (not corrected). It is very soluble in chloroform and ethyl acetate, but little if at all soluble in hot dilute hydrochloric acid.

Butyl alcohol extract. This extract was evaporated to dryness *in vacuo* in small portions. The residue was taken up with alcohol, filtered through a Büchner funnel and the insoluble portion washed with small quantities of hot butyl alcohol. The two alcoholic filtrates were evaporated *in vacuo* to dryness and the residue taken up with absolute alcohol. This treatment was repeated again; the insoluble portion was separated by filtration and the clear, red-brown, alcoholic solution was evaporated *in vacuo* until all the alcohol was removed. The residue was treated with warm water and a not inconsiderable amount of a brown product was removed by filtration on a Büchner filter. This residue was dissolved in absolute alcohol and the alcoholic solution evaporated *in vacuo* in tared dishes. The following percentages of the total nitrogen were thus obtained.

Time of hydrolysis in hours	12	24	48	96
	1·36	0·65	0·47	0·82

By treatment with ether part of this substance was dissolved. The ethereal solution evaporated in tared dishes gave the following results expressed as before in percentages of the total nitrogen.

Time of hydrolysis in hours	12	24	48	96
% N	0·70	0·36	0·21	0·56

This substance has all the properties of a ketopiperazine. It decomposes at 245° and then immediately volatilises.

The portion insoluble in ether was crystallised from absolute alcohol, from which it separated in fine hair-like needles; with the exception of the insolubility in ether it presented the same characters as the other substance. The ready solubility in ethyl acetate and chloroform, and its behaviour towards dilute solutions of acids and alkalis leave little doubt that it is a ketopiperazine.

Chloroform extract. A brown sticky mass was extracted by this solvent, which, owing to its semi-solid condition, was not weighed.

From these results it is evident that even after a prolonged hydrolysis ring compounds can be found amongst the products. But it is important to note that, in the case of the butyl alcohol extract, these compounds after reaching a minimum rapidly increase, thus showing that by prolonging the time of hydrolysis condensation takes place and this is confirmed by the increase of the nonamino-nitrogen.

SUMMARY.

1. The muscular tissue of the common cod was hydrolysed with sulphuric acid for varying periods. It was found that the percentages of basic nitrogen precipitated by phosphotungstic acid vary for a given period of hydrolysis with the conditions of precipitation, thus confirming the previous results obtained.

2. Evidence is given to show that even after a prolonged hydrolysis closed ring compounds or ketopiperazines are found in the hydrolysate, and that their percentage increases if the hydrolysis is prolonged.

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XLV. THE INFLUENCE OF THE HYPOPHYSIS ON METABOLISM, GROWTH AND SEXUAL ORGANS OF MALE RATS AND RABBITS.

II. INFLUENCE OF EXTRACTS OF HYPOPHYSIS ON THE BODY WEIGHT, WEIGHT OF FAT, OF SEXUAL ORGANS AND OF ENDOCRINE ORGANS OF RATS.

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(*Received February 28th, 1930.*)

HISTORICAL.

The hypophysis and fat deposition.

It is an established fact that patients with hypopituitarism and animals after the removal of the hypophysis develop a pronounced adiposity, while young animals in the same circumstances show, in addition, retardation of growth [see bibliography in Biedl, 1916; Barker, Hoskins and Palmer, 1922; Schaefer, 1926; Geiling, 1926].

In agreement with these observations the gaseous metabolism is found to be decreased [Narbut, 1903; Wolf and Sachs, 1910; Aschner, 1912, 1913; Aschner and Porges, 1912; Benedict and Homans, 1912; Foster and Smith, 1926]. It was, however, unexpected that injections of pituitary extract into animals should also produce adiposity [Delille, 1909; Evans and Long, 1922] and lowering of metabolism [Bernstein and Falta, 1912; Korenchevsky and Dennison, 1929].

The influence upon growth of the administration of the anterior lobe of the pituitary.

In spite of the large number of experiments which have been performed on mice, rats, guinea-pigs, rabbits, puppies and chicks, either fed or injected with extracts and preparations of whole hypophysis or of the anterior lobe, the most contradictory results have been obtained.

Increased growth of the animals was observed by Schaefer [1909], Goetsch [1916], Gudernatsch [1918], Klinger [1919], Marinus [1919] and Robertson and his co-workers [1916–1925]. Retardation or cessation of growth was obtained by Thompson and Johnston [1905], Cerletti [1907], Sandri [1907, 1909], Cushing [1909], Aldrich [1912, 1913], Wulzen [1914], Pearl [1916] and

Maxwell [1916]. Administration of pituitary preparations had practically no influence upon growth in the experiments of Lewis and Miller [1913], Drummond and Cannan [1922], Smith [1927, 2] and Reichert [1928]. Extremely elaborate experiments were performed by Evans and his co-workers [1921–1928]. They state that when an extract of the anterior lobe of the hypophysis is injected intraperitoneally into young rats from the day of weaning, acceleration of growth is produced, some of the treated rats being from 100 to 250 g. heavier than their normal litter mate controls at eight months of age. Oral administration is ineffective. No detailed account of these experiments has been published.

Smith, Walker and Graeser [1923], Smith and Graeser [1924], and Smith [1927, 1, 2, 3, 4] produced dwarfism by destroying the pituitary body in rats, and found that growth was resumed after injections of anterior hypophysial substance prepared by the method of Evans and Simpson [1926].

Van Wagenen [1927, 1928], using similar extracts of the anterior lobe, increased the body length and the weight of castrated rats up to those of the normal controls.

Putnam, Teel and Benedict [1928], who also used alkaline extracts of hypophysis, produced acceleration of growth in growing rats and one dog, but not in rabbits. The injections were given to the dog for many months before any effect was observed. In the hypophysectomised dog and the rats, restoration of growth was produced. They concluded that the growth of hair and the healing of wounds is much delayed after hypophysectomy, but these processes may be restored to normal by administration of the hormone.

The latest experiments by Larson, Bergeim, Barber and Fisher [1929] do not show any influence of similar extracts, injected intraperitoneally, on the growth of rats, whilst Hewitt [1929] confirmed the results obtained by Evans.

The influence of the administration of the hypophysis on the sexual organs of male animals.

A stimulating effect of pituitary feeding or injections of pituitary extracts on the sexual development or the size of testes was noticed by Wulzen [1914], Goetsch [1916], Klinger [1919] and Marinus [1919]. No such influence, however, was observed in the experiments of Pearl [1916], Maxwell [1916], Kross [1922] and Smith [1927, 3].

Evans and his co-workers [1921–1928] found that an extract of the anterior lobe of the hypophysis delays sexual maturity in the male rat. Autopsy of two treated male rats, when compared with their controls, showed great diminution of testis weights. Histologically, smaller tubules were found, but no evidence of any other degenerative changes [Evans and Simpson, 1926]. The authors succeeded in separating the principle in the anterior lobe affecting ovulation in rats from that controlling general body growth by the addition of alcohol to the extract until the concentration of alcohol reached 50 %. The supernatant fluid, after centrifugation, still possessed the principle affecting the gonads, but not that accelerating growth. Probably the latter principle had been carried down with the protein precipitate or been destroyed. Both hormones, however, are still active after bubbling oxygen through the extract for one hour or after adding alcohol to 8 % and keeping at room temperature for one week.

In a later paper Evans and Simpson [1928], concluded that there is present in the hypophysis not an ovary-depressing, but an ovary-stimulating hormone, which is required for the normal development of the ovaries, thyroid and suprarenal cortex. Their previous contradictory results are explained by the fact that the growth-promoting hormone completely nullified the effect of the gonad-stimulating hormone, when simultaneously administered. Alkaline aqueous extracts contain a growth-stimulating hormone and acid extracts of the anterior lobe a hormone provoking sexual maturity in young female rats. By these different methods of extraction these two hormones can be separated. Unfortunately, up to the present time no detailed account of these experiments has been published.

Zondek and Aschheim, in a series of papers [1927–1928] describe an ovary-stimulating hormone in the urine of pregnant women which they believe to be secreted by the anterior lobe of the hypophysis. These authors state that although a single injection of this substance is without effect on male mice, repeated injections, during a period of about 2 weeks, caused the secondary sexual glands, but not the testes, to become hypertrophied. The seminal vesicles were about five times larger than those of the control animals. This did not occur in castrated animals.

Fels [1927], injected infantile male mice with the serum from pregnant women. The testes were smaller than normal, but histologically the amount of the interstitial cells was increased. The prostate and seminal vesicles were hypertrophied, which latter changes were similar to those which occurred in the experiments of Zondek and Aschheim. In Biedl's [1927] experiments injections of hypophysial extract or emulsion retarded the development of the sexual system of male rats and mice, especially of the testes and seminal vesicles. The amount injected was from 0·05 to 0·25 g. of fresh glandular substance. Smith [1927, 3] injected rats with an extract prepared by the method of Evans and Simpson [1926] from ox pituitaries. These injections not only did not cause the restoration to normal of the atrophied testes of hypophysectomised rats, but even prevented their restoration by pituitary homotransplants, which were made simultaneously with the injections.

Larson, Bergeim, Barber and Fisher [1929] found that the weights of the testes of the rats receiving injections were less than those of the controls. However, they suggest that this may be attributed to the lower weights of these rats. The figures were not given.

The influence of the injections on the thyroids and adrenals.

Smith [1927, 3] observed in hypophysectomised rats atrophy of the suprarenal cortex, thyroids and parathyroids. Partial or complete repair was produced by daily homotransplants of fresh pituitaries, but intraperitoneal injections of extracts prepared by the method of Evans and Long, made from ox pituitaries, did not repair the atrophied thyroid or suprarenal cortex or the atrophied testes of hypophysectomised rats.

Loeb and Bassett [1929] prepared acid and alkaline extracts from dried and powdered anterior lobe of the pituitary of cattle and injected these intraperitoneally into guinea-pigs. After 7 to 19 days the injections resulted in hypertrophy of the thyroids. During the period of the injections the animals lost weight. The method of preparation suggests that probably the response of the thyroids was the typical reaction to injections of any emulsion of animal tissue contaminated with bacteria and prepared without sterile precautions.

The analysis of the papers, mentioned in this historical review, shows a perplexing discrepancy in results. It may be that the difference in the results obtained by different investigators is to be explained (1) by the presence in the anterior lobe of more than one hormone, some of these hormones being antagonistic in action; (2) by the different methods of preparation of the extracts; (3) by deficiencies in general technique in some investigations, especially the use of control animals not of the same litter and lack of control of the diet (its uniformity and its sufficiency in respect of all the factors necessary for growth of the animal in general and of some organs in particular).

THE PRESENT EXPERIMENTS.

Experiments were performed on 87 male rats, of which 76 were young growing rats and 11 were old rats. All the rats belonged to litters, bred at the Lister Institute, of which the day of birth was known. Each litter was divided into two groups, a control group, into which 20 % glycerol in saline solution was injected, and an experimental group into which extract of the anterior lobe of the pituitary was injected. The extracts were injected intraperitoneally, daily, or with some intervals of one, or rarely, of two, days. Seven litters were used for the injections without previous operation, a total of 23 rats; 27 rats belonging to another 7 litters were castrated and 37 from 9 litters were made cryptorchid. The experiments on cryptorchid rats are recorded in Tables I–III in two separate groups A and B, since the experiments on the rats of group B were performed later and with a different batch of the extract. Castrated and cryptorchid rats, having a decreased and an increased sexual inner secretion respectively, were used as well as normal rats since there is a close relationship between the pituitary and the sexual glands. Castration was performed in the usual manner. The animals were made cryptorchid by pushing back the testes from the scrotum into the abdominal cavity, occluding the inguinal canal and putting a suture on the basal portion of the *tunica vaginalis*. The method of preparation of the extract was almost the same as that used by Evans and Simpson [1928], which produced increased growth in rats. The only difference was that the glands were first ground with glycerol, instead of with water, since a glycerol extract is more easily kept in a fresh condition in the cold store than is a watery extract.

The detailed method of preparation of the extract is as follows. The anterior lobes of pituitary glands, obtained from the slaughter house on the day of killing of the cattle, were, with sterile precautions, chopped to a thin paste and then finely ground with sand in a mortar. Two volumes of glycerol were added and the glycerol paste was kept in the cold store at $-5°$. On the day of injection one part of the paste was mixed with $1\frac{1}{2}$ parts of distilled water and to this mixture $N/5$ NaOH was then added in an amount equal to three-eighths of the volume of the mixture. This was left to stand for about 20 minutes and then neutralised or made slightly acid with $N/5$ acetic acid. By the addition of a few drops of $N/5$ NaOH the p_{H} was brought to 7·4. The solution was then centrifuged and the supernatant fluid was used for the intraperitoneal injections, in amounts of from 0·5 to 2 cc. per rat. No abscesses or any toxic symptoms due to the injections were observed.

The animals were fed on a special paste, consisting of a normal synthetic diet, so as to ensure that the quality of the diet was identical for each animal. The amount of the diet consumed was recorded. The composition of the diet was the same as of that used for vitamin experiments and was described elsewhere [Korenchevsky, 1922, p. 7]. The duration of the experiments varied, for different litters, from 6 to 11 weeks.

In order to save space the detailed tables of the weights of each individual rat or organ are not given. The influence of the injections was in every case in the same direction as shown by the average figures, given in Tables I, II and III.

Table I. *The influence of intraperitoneal injections of anterior lobe extract on the weight of young and old rats.*

	Controls, glycerol-injected					Anterior lobe extract-injected				
		Weight in g.		Gain + or loss − in weight in g.			Weight in g.		Gain + or loss − in weight in g.	
Groups	No. of rats for averages	Init.	Final	Total	Per week	No. of rats for averages	Init.	Final	Total	Per week
Young rats:										
Normal	8	61·3	233·8	+172·5	+19·6	9	55·9	223·4	+167·5	+19·1
Castrated	12	80·8	214·8	+134·0	+17·0	15	78·6	202·9	+124·3	+15·8
Cryptorchid A	8	74·5	211·1	+136·6	+16·9	12	71·7	205·7	+134·0	+16·6
Cryptorchid B	4	53·5	228	+174·5	+18·6	8	53·0	240	+187	+19·8
Old rats:										
Normal	3	405·0	397·0	−8·0	−0·9	3	366·0	355·0	−11·0	−1·3
Cryptorchid	2	418·0	396·0	−22·0	−2·3	3	451·0	423·0	−28·0	−2·9

The influence on growth and appetite.

The results in relation to growth are given in Table I. The figures represent averages. These figures show no well-defined influence on the growth of the male rats injected with the extract, as compared with that of the controls injected with glycerol solution. The final weight and the gain in weight per week of both groups of young rats varied only slightly. Old rats, in both groups, lost weight a little during the period of the experiment. The variation in consumption of paste between the two groups was slight, being from 1 to 2 g. per day greater in about 65 % of the groups of the pituitary-injected rats.

Table II. *The influence of intraperitoneal injections of anterior lobe extract on the weight of "retroperineal fat" and "testicular fat" of rats.*

	Controls, glycerol-injected			Anterior lobe extract-injected		
Group of rats	No. of rats for averages	Actual weight in g.	Per 200 g. of rat weight in g.	No. of rats for averages	Actual weight in g.	Per 200 g. of rat weight in g.
		"Retroperineal fat."				
Normal	11	8·26	5·83	12	5·56	4·20
Castrated	12	6·60	5·99	15	4·37	4·02
Cryptorchid A	10	5·31	5·26	15	7·89	5·28
		"Testicular fat."				
Normal	11	8·01	5·61	12	5·58	4·24
Cryptorchid A	10	7·55	5·61	15	5·72	4·22
	Total "intra-abdominal fat" (*i.e.* Retroperineal + testicular fat).					
Normal	11	16·27	11·44	12	11·14	8·44
Cryptorchid A	10	12·86	10·87	15	13·61	9·50
Cryptorchid B	4	12·38	10·84	8	9·98	8·29

Table III. *The influence of intraperitoneal injections of anterior lobe extract on the average weight of the sexual organs and of the glands of inner secretion.*

		Controls, glycerol-injected		Anterior lobe extract-injected	
Organ	Group of rats	Actual weight of organ in g.	Weight in g. per 200 g. of rat weight	Actual weight of organ in g.	Weight in g. per 200 g. of rat weight
Testis	Normal	2·826	2·126	2·346	1·876
	Cryptorchid A	1·037	0·926	0·669	0·572
	,, B	0·784	0·689	0·628	0·528
Penis	Normal	0·310	0·222	0·284	0·222
	Cryptorchid A	0·262	0·222	0·179	0·144
	,, B	0·289	0·254	0·223	0·186
	Castrated	0·074	0·071	0·078	0·077
Prostate with seminal vesicles	Normal	1·960	1·386	1·389	1·068
	Cryptorchid A	1·214	0·999	0·386	0·321
	,, B	1·282	1·124	0·598	0·499
	Castrated	0·080	0·076	0·093	0·095
Adrenals	Normal	0·0636	0·0474	0·0620	0·0494
	Cryptorchid A	0·0616	0·0523	0·0639	0·0528
	,, B	0·0509	0·0449	0·0703	0·0589
	Castrated	0·0690	0·0644	0·0679	0·0682
Thyroids	Normal	0·0382	0·0271	0·0278	0·0218
	Cryptorchid A	0·0336	0·0272	0·0264	0·0215
	,, B	0·0216	0·0190	0·0201	0·0167
	Castrated	0·0241	0·0227	0·0217	0·0217
Hypophysis	Normal	0·0114	0·0085	0·0097	0·0077
	Cryptorchid A	0·0115	0·0098	0·0102	0·0085
	,, B	0·0086	0·0075	0·0095	0·0079
	Castrated	0·0126	0·0115	0·0116	0·0116

The influence on fat deposition.

Since the separation and weighing of the whole of the fat is very difficult in rats, the weight of the intra-abdominal fat was taken as an indication of the general development of the fat in the body. In rats part of this fat is easily separated from the retroperineal region ("retroperineal fat" in the tables). In castrated rats only this fat was taken for weighing. In normal and cryptorchid animals, in addition, the fat attached to the testes was separately weighed ("testicular fat" in the tables). Both fats taken together will be referred to as "intra-abdominal fat." The average results are given in Table II. In this table the weight of fat of young growing rats is not separated from that of old rats, since the fat deposition was similar in both groups. Comparison of these average figures, especially taking into consideration those reduced to 200 g. of rat weight, shows a decreased amount of fat in the rats injected with the extract of the anterior lobe. Detailed figures of the weight of fat of each individual rat (which are not given in order to save space) as well as the average figures in Table II show that this decrease was absent only in the case of the retroperineal fat of Group A of cryptorchid rats. The testicular fat of cryptorchid rats was, however, decreased; so that the total amount of intra-abdominal fat, calculated per 200 g. of rat weight, was decreased by the influence of injections of anterior lobe extract in all three groups of rats, normal, castrated and cryptorchid. Per 200 g. of rat weight, the average amount of intra-abdominal fat in the injected rats was less by 12·6 to 33 % as compared with that in the respective groups of control animals.

The influence on sexual organs.

The results are given in Table III and represent the average weights in g. of testis, prostate with seminal vesicles, and of penis. The number of rats used for the average was in each group the same as that in Table II. The most suitable figures for comparison are those calculated per 200 g. of rat weight. They show a striking decrease in weight of the sexual organs in cryptorchid animals under the influence of anterior pituitary injections. For example, the weight of the prostate and seminal vesicles was about 68 % less than that of control rats in group A and about 56 % less in group B. The corresponding figures for penis and testis were 35 % and 38 % less than the controls in group A; in group B of cryptorchids the respective figures were 27 and 23 % less than those of the controls. In normal animals the decrease in the weight of the prostate with seminal vesicles and of the testes of rats injected with pituitary was much less pronounced than those of cryptorchid rats. The respective figures for the decrease of weight of these organs, compared with the controls, were about 23 and 12 %. There was no change in the weight of the penis.

In castrated animals the influence of pituitary injections was not clear. Although the penis, prostate and seminal vesicles were on the average heavier than in the controls, it is impossible to draw definite conclusions from these figures, since the individual weights of these organs, included in the average, varied considerably. This was due, firstly, to the difficulty of accurate separation of fat from the very atrophied and degenerated sexual organs of castrated rats; and secondly, probably to the absence of considerable changes in the organs of castrated animals under the influence of pituitary injections. That the injections had the maximum effect on cryptorchid animals shows that the extracted hypophyseal substances are closely correlated with a special testicular tissue. However it is not possible from the present data to conclude whether this effect was due to the atrophy of the generative tissue or to the hypertrophy of the interstitial tissue.

The influence on the adrenals, thyroids and hypophysis.

It is possible to see from Table III definite decrease only in the weight of the thyroids of normal and cryptorchid animals injected with anterior lobe (on the average, of about 20 % in normal rats and in group A and about 12 % in group B below that of the controls). In castrated rats the thyroids are already atrophied, as a result of castration. The injections of anterior lobe extract did not increase this atrophy in all the castrated rats. Of the 15 castrated rats there was a decrease in weight of the thyroid in only 7 rats, in 5 rats there was no change and in 3 the gland had increased in weight as compared with the castrated controls. The divergence of these results, therefore, renders them inconclusive in considering castrated rats. Similar divergent results were obtained in the weights of the hypophysis in the individual rats used in this experiment. On the average, however, there was a slight decrease in the weight of this gland in the groups of normal and group A of cryptorchid rats (about 9 and 13 % respectively below that of the controls).

The weights of the adrenals did not show any definite change under the influence of anterior lobe injections in the normal and castrated rats and in group A of cryptorchid rats. However, there was an increase by about 31 % in the weight of the adrenals of the rats of group B, injected with a different batch of the pituitary extract.

DISCUSSION.

Taking into consideration our previous paper [Korenchevsky and Dennison, 1929] as well as the results obtained in the present experiments, it is possible to state that from the anterior lobe of the hypophysis an extract was prepared which depressed nitrogen metabolism, produced atrophy of the sexual organs (testis, prostate, seminal vesicles and penis) and of the thyroid gland in male rats. This extract did not affect the growth of rats but it decreased the amount of body fat, as judged by the amount of intra-abdominal fat. The atrophy of the sexual organs was more pronounced in the cryptorchid group than with the normal and castrated rats. With the exception of a considerable decrease of body fat, the smallest changes were observed in the group of castrated rats. These results, therefore, agree with those of Evans and Simpson [1926], Smith [1927, 3] and Biedl [1927], whose extract depressed the development of the testes. Smith, alone, made accurate weighings of the glands of the few male rats used in his experiments.

In the numerous experiments on feeding with, or the injection of, pituitary preparations performed by other investigators no observation apparently was made of the amount of the body fat of the experimental animals. In no case, including the experiments of Evans and his co-workers, was a direct weighing made of the fat in the body of the animals.

To my surprise I was unable to obtain the stimulation of body growth mentioned by Evans and Simpson [1928], although my alkaline extract was prepared in a manner nearly identical with theirs. It is improbable that the use of glycerol in place of water in the first stage of the preparations (see p. 387) was responsible for the divergence. Since the influence on the sexual organs was a depressing and not a stimulating one, an antagonistic action of the sexual hormone upon the growth-promoting hormone cannot be the explanation as was suggested by Evans and Simpson for their results [1928]. Neither from the papers of Evans and his co-workers, nor from the papers of other authors, can indisputable evidence be obtained of the presence of hormones in extracts of hypophysis which stimulate the growth of male sexual organs. The chief difficulty in interpreting many of the experiments is that the authors do not always give figures for the weight of the testes and do not calculate their results per unit of body weight, nor do they use for their controls in their experiments brothers from the same litter. These deficiencies in technique may lead to the gravest mistakes, since I have observed that different litters have different weights of organs and of body fat.

It is important to stress, however, that on one hand Smith's [1927, 3, 4] implantation experiments showed a stimulating influence of the anterior lobe on the male sexual organs, adrenals and thyroids, and Evans and his co-workers obtained extracts which were growth-stimulating. On the other hand, extracts can be obtained, as my experiments show distinctly, which depress nitrogen metabolism, diminish the size of the testes, the prostate, the seminal vesicles, the penis and the thyroid and decrease the deposition of fat. Nevertheless, these extracts do not influence body growth. It may, therefore, be suggested that probably the pituitary contains antagonistic substances for all these functions. It would be unjustifiable, as Evans and Simpson [1928] do, to suggest that there are only two hormones, one required for the normal development of the gonads, thyroid and adrenals and a second for the promotion of growth, and that the latter can be completely nullified by the simultaneous administration of the first.

The extract which I isolated seems to differ from that obtained by Evans and his co-workers, since in my experiments a growth-promoting substance was absent or nullified and at the same time evidence was lacking of the presence of a hormone which stimulates the activities of the gonads. On the contrary, the extract possessed, except any influence on body growth, a general depressing action.

SUMMARY.

1. A slightly alkaline extract, p_H about 7·4, was prepared from the anterior lobe of the pituitary of oxen which had been ground with glycerol, kept at – 5° and injected intraperitoneally into normal, cryptorchid and castrated young and adult male rats in amounts of 1 to 2 cc.
2. No decided influence was noticed on the growth of the animals as judged by the weight of the rats.
3. Body fat as judged by the intra-abdominal fat was decreased on the average by about 24 %.
4. Under the influence of injections of the extract the weight of penis, testes, and prostate with seminal vesicles was decreased to the greatest extent in cryptorchid animals (on an average for these organs by 31, 30 and 62 % respectively). This effect was less noticeable in normal and absent in castrated rats, showing that a correlation exists between the extracted hypophyseal substances and a special testicular tissue.
5. The weight of the thyroids was decreased by about 17 % in normal and cryptorchid animals, but not significantly in castrated rats.
6. It is concluded that the anterior lobe contains substances diminishing the weights of the penis, prostate, seminal vesicles, testis and thyroids and decreasing fat deposition and nitrogen metabolism.
7. It is suggested that these substances are antagonistic to the growth- and male gonad-stimulating hormones, made evident by implantation experiments; and to the growth-promoting hormone, prepared by Evans and his co-workers.

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XLVI. THE HAGEDORN-JENSEN METHOD APPLIED TO VARIOUS SUGARS.

RELATION OF REDUCING POWER TO CONFIGURATION.

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The increasing knowledge of sugar structure and its physiological significance has prompted a close scrutiny of the methods for the quantitative analysis of reducing sugars. Most of the methods are based on the oxidation of the sugars. The amount of oxygen consumed permits a comparison of the oxidising reagents. The products of oxidation are in most instances not uniform, thus the oxygen factors are only pseudo-stoichiometrical [Sobotka, 1926], but a comparison of these factors with the degree of oxidation in the possible reaction products conveys a general idea of their nature.

Lobry de Bruyn and Nef and, particularly, Evans [1929] have dealt with the numerous products obtained by alkaline rearrangement and oxidation of carbohydrates. These researches have established beyond doubt what may be termed progressive enolisation as the key to sugar destruction. By extending the range of substrates over the field of methylated glucoses it has been demonstrated [Sobotka, 1926] that the methoxy-group is an obstacle in the progress of enolisation in the Bertrand solution and more so in the less alkaline Fehling reagent.

Purely steric differences, as between glucose and galactose, give rise to merely insignificant deviations with the usual alkaline copper solutions. Micromethods like Bang's according to Rohny [1928] and the Folin-Wu method according to Greenwald *et al.* [1924] yield rather divergent figures for hexoses other than glucose. It has been found that alkaline mercury solutions oxidise quantities of aldoses and ketoses above 10 mg. at a remarkably constant rate of 6·0 oxygen equivalents. This linear function was entirely distorted for smaller amounts *ceteris paribus* (unpublished experiments). Such irregularities in the lowest sections of the sugar-oxygen curves are at least in part due to a passive state of the complex metal ion in absence of certain organic groups; their bearing upon steric differences of the sugars is important but not obvious.

A constant difference between glucose and galactose was realised over a wide range of concentrations when employing potassium ferricyanide as the oxidising agent. The Hagedorn and Jensen method has been adapted to amounts of glucose of 1–10 mg. by Issekutz and Both [1927] and up to 4 mg. by Hanes [1929]. We adopted Hanes's accurate and convenient volumetric procedure. Pucher and Finch [1928] seem to have been the first to describe the low reduction power of galactose with the Hagedorn-Jensen reagent. Their results were recently confirmed by Hawkins [1929] for the gasometric ferricyanide reagent.

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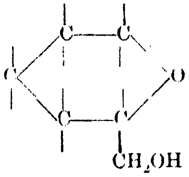
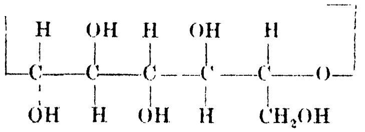
[1] Isadore Hernsheim Research Fellow.

The complex iron salt of the mildly alkaline Hagedorn-Jensen reagent probably approaches the conditions of physiological sugar oxidation more than any copper, mercury, bismuth or permanganate solution. One may attribute special significance to its steric predilections.

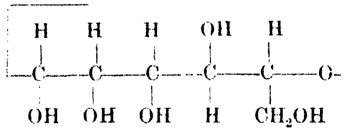
By unrolling the cylinder of the accepted pyranoid formulas of the hexoses [Haworth, 1929], we obtain the following symbols for the *d*-hexoses:



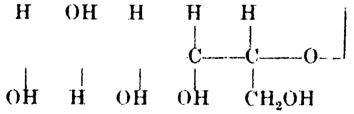
I.



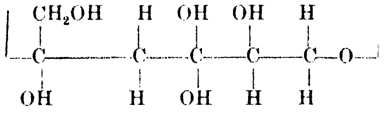
II. β-Glucose



III. β-Mannose



IV. β-Galactose



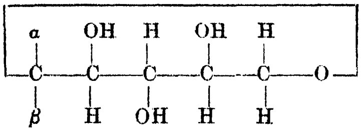
V. β-Fructose

Disregarding the labile steric configuration on the first C atom (C_1) glucose shows an alternation of the hydroxyls throughout the molecule: C_2 *trans* C_3 *trans* C_4. Accordingly in mannose and rhamnose: C_2 *cis* C_3 *trans* C_4, and in galactose: C_2 *trans* C_3 *cis* C_4.

The average reducing power of mannose is only slightly less than that of glucose. The isomerism along the C_2—C_3 link is therefore not important for the stability of the hexose chain towards ferricyanide. In all other instances the increased reactivity of the *trans*-configuration is conspicuous. The great difference of approximately 24 % for glucose minus galactose must be attributed to the *trans*-configuration on C_3—C_4.

Haworth [1929] pointed out that the asymmetry of the fifth carbon atom may be interpreted in the above ring formula (I) as a *trans* or *cis* location of the side chain —CH_2OH with respect to the hydroxyl on the fourth carbon atom. It is not possible to ascertain the influence of this isomerism as the number of hexoses available for comparison is limited; but it seems probable that this end of the carbon chain is of no importance for the reducing power.

In order to extend this investigation we tested three of the four possible aldopentoses.



VI.

d-Xylose (the Greek letters indicate the location of OH on C_1 in the isomeric forms)

Omitting the *cis-trans*-isomerism between C_1 and C_2 (α–β forms), they show the following differences:

Xylose (VI)	C_2 *trans* C_3 *trans* C_4
Arabinose	C_2 *trans* C_3 *cis* C_4
Ribose	C_2 *cis* C_3 *cis* C_4.

Arabinose with an oxygen equivalent for ferricyanide of 4·18 resembles galactose with 4·04; xylose with 4·56 equivalents ranges below the analogous hexose, glucose, with 5·31. The decrease of reducing power by 8·3 % and 7·3 % from xylose to arabinose to ribose permits the conclusion that in the oxidation of aldopentoses, the influence of *trans* or *cis* position between C_2 and C_3 is equal to that between C_3 and C_4. This finding, together with the lower absolute reducing power of pentoses, shows that the progress of enolisation and oxidation does not reach beyond C_3 in the five-carbon sugars under the experimental conditions. The oxygen equivalent of ribose of less than 4 (= two atoms oxygen) barely allows for the formation of a keto-pentonic acid and for the cleavage into formic acid and erythronic acid. This is in sharp contrast to the pivotal C_3—C_4 link of the hexoses.

The Lobry de Bruyn rearrangement leads to the formation of talose and tagatose from galactose. Determinations of optical rotation, copper and ferricyanide reduction from Lobry de Bruyn equilibria of glucose and of galactose reveal parallel changes. Hence, in the ketohexoses the reducing power of tagatose (C_3 *cis* C_4 *trans* C_5) is essentially lower than that of fructose (V) (C_3 *trans* C_4 *cis* C_5). This corroborates the assumption that the considerable differences in the reducing power of hexoses are due mainly to the *cis-trans*-distribution of the hydroxyls on the third and fourth carbon atom.

The reducing power of rhamnose is identical with that of glucose or mannose in amounts of 2 mg. or less; in higher concentrations, it exhibits considerable deviations.

Despite the identity of their reducing moiety we observed a slight increase of maltose over lactose. This may be due to the difference of the other moiety set free by partial hydrolysis which is in turn indicated by the high absolute equivalent of more than 8 equivalents of oxygen.

The difference between the reducing power of glucose and galactose with respect to $K_3Fe(CN)_6$ may be applied with great advantage to the analysis of mixtures, as in selective glycolysis, in the hydrolysis of saponins, etc. In combination with the polariscope, the accuracy of these analyses proved to be almost doubled.

Table I. *Titration data and oxygen equivalents.*

Sugar mg.	Glucose cc.	Fructose cc.	Mannose cc.	Galactose cc.	Glucose + Galactose cc.	Glucose + Fructose cc.	Xylose cc.	Arabinose cc.	Ribose cc.	Rhamnose cc.	Maltose cc.	Lactose cc
					0·01 N thiosulphate							
0·5	1·45	1·55	1·45	1·02	1·21	—	1·55	1·40	1·25	1·63	1·33	1·22
1·0	2·91	3·10	2·92	2·19	2·46	2·88	3·11	2·72	2·55	3·13	2·44	2·44
1·5	4·40	4·70	4·30	3·40	3·75	4·34	4·65	4·15	3·79	4·86	3·63	3·49
2·0	5·91	6·28	5·87	4·50	5·00	5·87	6·07	5·55	5·11	6·49	4·96	4·54
2·5	7·38	7·83	7·46	5·64	6·33	7·32	7·49	7·05	6·39	7·67	6·08	5·78
3·0	8·93	9·39	8·63	6·86	7·65	8·87	9·06	8·41	7·76	9·27	7·23	6·89
3·5	10·55	10·90	10·38	8·12	9·24	10·36	10·59	9·92	9·14	10·51	8·47	7·92
4·0	—	12·48	11·42	9·12	10·49	11·91	11·83	11·28	10·49	11·56	—	8·88
4·5	—	—	—	10·41	—	—	—	12·29	11·76	12·10	—	9·92
					Oxygen equivalents							
0·5	5·22	5·58	5·22	3·67	4·35	—	4·65	4·20	3·75	5·35	9·58	8·80
1·0	5·26	5·58	5·26	3·94	4·43	5·19	4·66	4·08	3·82	5·14	8·79	8·79
1·5	5·28	5·64	5·16	4·08	4·50	5·21	4·65	4·15	3·79	5·31	8·72	8·38
2·0	5·32	5·65	5·28	4·05	4·50	5·28	4·55	4·16	3·83	5·32	8·92	8·18
2·5	5·31	5·62	5·36	4·06	4·56	5·28	4·49	4·23	3·83	5·04	8·76	8·33
3·0	5·36	5·63	5·18	4·12	4·59	5·32	4·53	4·21	3·88	5·07	8·68	8·27
3·5	5·42	5·61	5·32	4·15	4·73	5·33	4·54	4·25	3·89	4·91	8·71	8·14
4·0	—	5·62	5·14	4·11	4·72	5·37	4·44	4·23	3·93	4·67	—	7·99
4·5	—	—	—	4·16	—	—	—	4·10	3·92	4·40	—	7·94
Average	5·31	5·62	5·24	4·04	4·55	5·28	4·56	4·18	3·85	—	—	—
% deviation from glucose	0	+5·8	-1·3	-23·9	-14·3	-0·5	-14·1	-21·3 (-8·3)*	-27·5 (-15·6)*	—	—	—

* % deviation from xylose.

EXPERIMENTAL.

The procedure of Hanes was followed, but 0·01 N sodium thiosulphate was used for all titrations. In Table I are given the titration data of duplicate determinations and the oxygen equivalents for the sugars studied. All sugars, except the two disaccharides, were anhydrous; maltose and lactose were the monohydrates. The stock solutions were freshly prepared and checked by the Bertrand method and by optical rotation. These concentrations were 10·00 ± 0·03 %. Decimal dilutions were made for the determinations and any deviation from 10·00 % in the stock solution was taken into account. The variation in reducing power with concentration of sugar is shown by the curves in Fig. 1.

10 % solutions of glucose and galactose were set up in 0·1 N NaOH; one portion of each was neutralised immediately, another after 24 hours at room temperature. In another experiment they were exposed for 12 hours to 0·5 N NaOH at 30°. The $[\alpha]_D$ of the glucose changed from + 52° to + 11° in the former and to − 4° in the latter experiment, the $[\alpha]_D$ of the galactose from + 81° to + 56° and + 43° respectively. The apparent diminution of ferricyanide-reducing power was 5·4 % for glucose and 7·8 % for galactose in the second experiment, whereas the 0·1 N alkali did not decrease the reducing titre of either solution for ferricyanide. As fructose shows a smaller reduction than glucose by Bertrand's method [Rohny, 1928] but a greater one with ferricyanide, the

quotient $\frac{\text{Hanes}}{\text{Bertrand}}$ changes from 1·00 to 1·035 and 1·09 in the glucose experiments, and from 1·00 to 1·055 and 1·07 in those with galactose. From these findings it may be concluded that the Lobry de Bruyn alkali rearrangement of galactose into the epimeric talose and the ketose tagatose causes merely slight changes of ferricyanide-reducing power analogous to those observed in the glucose-mannose-fructose group. The average ferricyanide-reducing power of the alkaline equilibrium mixture derived from galactose remains more than 20 % below that from glucose.

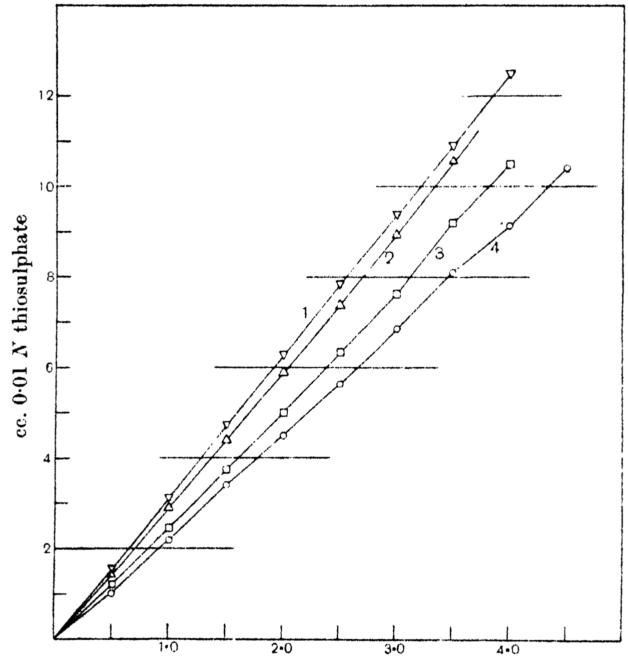


Fig. 1. Titration data for (1) Fructose (▽), (2) Glucose (△), (3) Galactose + glucose (1 : 1) (□) and (4) Galactose (○).

The reducing power of invert sugar is practically that of glucose instead of the expected average of glucose plus fructose. This parallels Rohny's [1928] findings for the Bertrand method. For the glucose-galactose mixture the curve approaches the mean value, but is slightly closer to the galactose curve. It is easy to correct the interpolations incident to the analysis of varying mixtures.

SUMMARY.

1. Significant differences in the reducing power of several sugars with $K_3Fe(CN)_6$ are demonstrated.

2. These differences are correlated with *trans* and *cis* configuration of the sugars in the following manner. In aldo- and keto-hexoses the configuration

between the third and the fourth carbon atom is the determining factor: in aldopentoses the configurations of C_2—C_3 and C_3—C_4 share the influence on the reducing power.

This is illustrated by the following examples:

glucose/mannose, isomerism on C_2—C_3, no difference in reducing power;	
glucose or mannose/galactose	C_3—C_4, *trans* > *cis*;
arabinose/ribose	C_2—C_3, *trans* > *cis*;
xylose/arabinose	C_3—C_4, *trans* > *cis*.

We wish to thank Dr P. A. Levene of the Rockefeller Institute for Medical Research, for a specimen of pure ribose.

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XLVII. A NOTE ON THE OXYGEN CONSUMPTION OF NERVE IN THE PRESENCE OF GLUCOSE AND GALACTOSE.

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(*Received February 28th, 1930.*)

In a previous communication [Sherif, 1930] reference was made to the effect of galactose on the oxygen uptake of mammalian (rabbit) peripheral nerve, as measured in Barcroft's apparatus. Since then, a good many more experiments have been performed, and the results, though at present isolated observations, seem worth recording briefly, in the expectation that they may have a bearing on future work.

It was found that, if galactose in concentrations of 0·1 to 0·25 % was added to the fluid (Ringer or Ringer plus phosphate buffer) in which one of the nerves under observation was bathed, the oxygen uptake, measured over the usual three hour period, was very nearly linear, while that of the "control" nerve (no galactose) usually showed a distinct falling off. If glucose were substituted for galactose, the same effect could not be demonstrated with certainty. Since, however, in a number of experiments, the oxygen uptake of the control nerve was itself almost linear, a certain doubt was thrown on the reality of the result, although the average values for oxygen uptake, calculated for a number of experiments, substantiated the findings. By prolonging the experiments over periods of 9 hours it has been possible to obtain definite results; errors due to bacterial growth in these experiments were avoided by washing the nerve and renewing the fluid at intervals of somewhat less than 3 hours. In these circumstances, a very definite falling off of the rate of oxygen uptake by the "control" nerve occurs, while that of the nerve in the presence both of galactose and glucose remains linear (Figs. 1 and 2).

Gerard [1927] found that glucose in concentrations of 0·1 % had no effect on, or in greater concentrations diminished, the oxygen uptake of frog nerve. Such would have been our own conclusion for rabbit nerve had we not finally prolonged our experiments. Holmes and Gerard [1929, 1930] have found that carbohydrate disappears from nerve during rest in oxygen, though the nature of the carbohydrate remains uncertain. Nerve contains at least 5 mg. of galactose per g. tissue, combined as cerebroside. If this carbohydrate were available for oxidation, it is hard to see why further additions should affect the oxygen uptake.

Loebel [1925] has found that, while the oxygen uptake of slices of brain tissue is very markedly increased by glucose, it is diminished by galactose. We have confirmed this effect with glucose, and we find that, provided Ringer's solution is used as suspension medium, galactose does not affect the oxygen uptake.

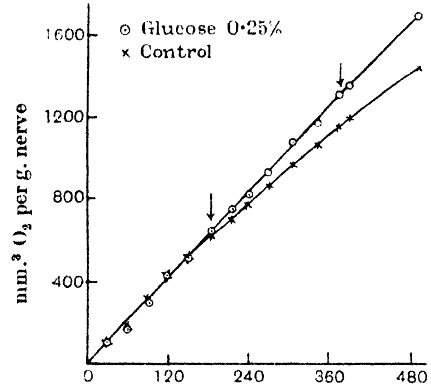

Fig. 1.

Time (minutes)

Fluid—4 parts Ringer, 1 part phosphate buffer, p_H 7·6.

Arrows indicate where fluid was renewed.

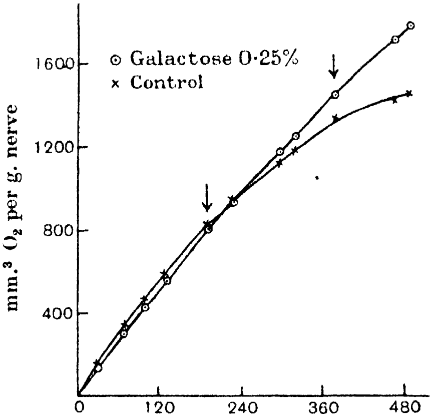

Fig. 2.

Time (minutes)

Fluid—4 parts Ringer, 1 part phosphate buffer, p_H 7·4.

Arrows indicate where fluid was renewed.

The quantitative difference in the behaviour of brain and nerve towards the two sugars is thus very striking.

SUMMARY.

Both galactose and glucose prolong the period for which isolated mammalian nerves take up oxygen at a steady rate.

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XLVIII. STUDIES ON PECTIN.

IV. THE OXIDATION OF PECTIN BY FENTON'S REAGENT AND ITS BEARING ON THE GENESIS OF THE HEMICELLULOSES.

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(*Received March 3rd, 1930.*)

It is now some time since Nanji, Paton and Ling [1925] showed that the constitution of the unit of the pectin molecule might be expressed by a definite chemical formula. Their views seem to have met with fairly general acceptance, as evinced by a number of later publications by various authors. Little attempt, however, has been made subsequently to examine the consequences following the treatment of pectin as a definite chemical entity. A number of researches has been carried out on the condition and function of pectin in the plant tissues, while the metabolic changes undergone by the pectic substances during the ripening and senescence of fruits have received particular attention; but in most cases the physiological aspect has been predominant, to the neglect of the chemistry of the subject. This is unfortunate, since it is not possible to obtain a complete and true picture of the metabolism of any substance until the chemical changes which result in its formation and which occur in its degradation have been elucidated. Neither of these conditions is yet satisfied in the case of pectin, since there is no information as to its genesis, and little concerning its mode of breakdown and ultimate degradation products. The purpose of this investigation was to isolate certain degradation products of pectin by means not dissimilar to those possibly obtaining in the living plant.

Candlin and Schryver [1928] recognised the close generic relationship between pectin and the hemicelluloses, and proposed to include such substances under the general term polyuronides. They claimed to have converted pectin to hemicellulose by more or less prolonged treatment with caustic alkalis, and made the suggestion that the process of lignification involved the transformation of pectin to hemicellulose and subsequently, perhaps, to lignin by some synthetic process. Attempts by one of us to repeat the experiments of Candlin and Schryver have, up to the present, resulted only in failure; the alkali produced only much charred material at temperatures approaching 100° and effected no appreciable change in the pectin at lower temperatures.

Nevertheless, the suggestion that pectin is converted to hemicellulose is an attractive one, based as it is on the fact that in non-lignified tissues cellulose and pectin are found, together with only small quantities of hemicellulose, whereas in lignified tissues we find cellulose, hemicelluloses and lignin, but only traces of pectin [O'Dwyer, 1925, 1926, 1928; Norris and Schryver, 1925].

The mechanism in the plant of the transformations indicated above is obscure, and few attempts have been made in the laboratory to imitate such changes. It would seem that the action of caustic alkalis is rather drastic, nor does it approximate to any action likely to take place in the living organism. A process of mild oxidation might, however, effect the conversion of pectin to an allied substance both in the plant and under laboratory conditions. Accordingly, attempts were made to treat pectin with suitable oxidising agents, and bromine in the presence of calcium carbonate was first employed, with indifferent success. The oxidation was extremely slow, and the products obtained were difficult to isolate owing to the presence of unchanged pectin. Later, the mild oxidation effected by Fenton's reagent—hydrogen peroxide in the presence of ferrous iron—suggested itself, and this proved to be the more useful reagent.

It will be recalled that the pectin formula of Nanji, Paton and Ling [1925] involves a unit consisting of one molecule of arabinose, one molecule of galactose and four molecules of galacturonic acid combined as anhydrides in a closed ring, the four carboxyl groups being free. From primary considerations it appeared possible that these carboxyl groups might be removed by the action of Fenton's reagent, and if at the same time the ring suffered no rupture then a product of the nature of a hexo-pentosan might be obtained.

As will appear in the experimental section, it was found that the action of Fenton's reagent was more vigorous than anticipated. The reaction was difficult to control; at temperatures below 30° no action was evident, whilst above 35° the pectin appeared to be oxidised completely away. Once commenced at 30° the action was strongly exothermic, control of temperature was sometimes difficult and if the temperature rose above 35° the yield of products precipitable by alcohol was considerably lowered.

Products in the form of white powders were obtained in each of a series of oxidations carried out on similar samples of a commercial pectin obtained from lemons and on calcium pectate similarly derived. These products are precipitable by alcohol and bear a strong physical resemblance to the hemicelluloses. Their almost complete lack of reducing power and their rather high rotatory power suggests that they are polymerised substances, and the results of analysis show that they are mixtures of substances whose simplest units consist of galactose and galacturonic acid combined in varying proportions. This result is a very surprising one and the original suggestion, that the acid groupings might be eliminated by Fenton's reagent, breaks down. The pectin ring appears to be broken, arabinose is lost and galactose and galacturonic acid remain. The reactions involved are probably much more complex than indicated by the foregoing, but at present no information is available which throws light on any intermediate stage of the completed reaction.

EXPERIMENTAL.

Preparation of Fenton's reagent.

The catalyst employed in this reaction is ferrous acetate, and since this is an unstable substance which readily oxidises in the air it is necessary to prepare it as required. About five parts by weight of barium acetate and three parts of ferrous sulphate are dissolved in a small volume of cold water. The filtered solution is employed in the reaction, but since the mixing has to be carried out in the cold, the precipitated barium sulphate is in such a fine condition that it passes good filter papers to a considerable extent. As will be seen later, the presence of barium sulphate tends to raise the ash content of the final product to a rather high figure, but this cannot easily be avoided. In some cases the barium sulphate was separated at the centrifuge, but this was not very satisfactory owing to partial oxidation of the ferrous acetate.

Oxidation of pectin.

The pectin employed in this work was a commercial product of good quality, with an ash content of 2·2 %, prepared by the method of Wilson [1925]. One litre of a smooth solution of 2 % concentration is placed in a large beaker and to this is added the iron catalyst as prepared by the method indicated above. For 20 g. of pectin approximately 0·2 g. of ferrous iron is sufficient. The beaker is placed in a water-bath at 30° and throughout the reaction the mixture is stirred as rapidly as possible with a strong stirrer provided with vanes. After the addition of iron, which causes the solution to stiffen and gel, 50 cc. of hydrogen peroxide (20 vols.) are run in at once, after which the viscosity of the solution rapidly diminishes. During the next 2 hours a further quantity of 150 cc. of hydrogen peroxide is added slowly from a dropping funnel and large quantities of carbon dioxide are evolved. Unless precautions are taken the temperature of the solution may rise to 50° or more and this must on no account be permitted since it is found that the yield is much decreased if the temperature is allowed to exceed 35°. The surrounding water-bath has therefore to be cooled at this stage of the reaction, although unless the temperature of the solution is initially raised to about 30° the reaction does not proceed to any extent. At the end of 4 hours the solution is filtered and poured into 2 to 4 volumes of alcohol, in which a white precipitate appears. This should be filtered off within a few hours, as the precipitate becomes greyish after longer contact with alcohol. A further small quantity of the product may be obtained by concentrating the alcoholic filtrate under diminished pressure and pouring into alcohol; this product is identical with the bulk of the precipitate. The total yield is small, about 5 % on an average.

Since the Fenton reaction for the degradation of sugars of one series to those of a lower one is usually carried out on the calcium salt of the sugar acid, calcium pectate, in the form of a gel in aqueous suspension, was employed in some cases in lieu of the pectin solution. The calcium pectate gel is prepared by adding to a solution of pectin an equal volume of saturated lime water. After standing overnight, the resulting gel is thoroughly washed with water and suspended in water for the oxidation, which is carried out as previously described. By using the calcium gel as the starting point, the high viscosity which is given by the pectin solution in the early stages is avoided. However, there seemed to be no particular practical advantage in this, and further there was the grave disadvantage that even more inorganic material was introduced, with a resulting effect on the ash content of the final product.

Many samples were prepared under various conditions of time, temperature and concentration of pectin and peroxide, but the conditions indicated previously give the best yield of product, careful control of temperature being of major importance. If the reaction is unduly prolonged the oxidation proceeds to a stage at which no precipitate is given with alcohol. The precise nature of the products under these conditions has not been determined but it is believed that the pectin is completely oxidised to carbon dioxide and water.

The ash content of all preparations was high, due to the ash of the original pectin and the barium sulphate added with the catalyst. Various attempts were made to lower the ash content. Slight acidification of the dissolved product with hydrochloric acid, followed by dialysis, was unsuccessful since it was found that the products passed through good quality parchment, resembling in this respect certain water-soluble hemicelluloses. By repeated solution in slightly acidified warm water, filtration and reprecipitation the ash content was reduced. The amount of ash remaining, however, was even then quite appreciable; one sample, for instance, was dissolved and reprecipitated many times, but still retained nearly 8 % of ash.

Hydrolysis of the oxidation product.

An examination of the products of hydrolysis was carried out in some detail. A small quantity of the preparation was boiled for several hours with 1 % sulphuric acid and neutralised while still hot with barium carbonate. The precipitated barium sulphate and excess barium carbonate were filtered off and the filtrate divided into three portions.

(i) Phenylhydrazine was added and the osazone prepared in the usual manner. The purified osazone was obtained in the form of crystals which resembled those described for galactosazone, when examined microscopically. Recrystallised from 60 % alcohol, the osazone had M.P. 181°; this is not in very close agreement with the recognised melting point of galactosazone—

191°—but the presence of small quantities of organic material may cause a lowering of the observed melting point.

(ii) Nitric acid was added to give a concentration of about 25 % and the mixture gently heated on a water-bath for several hours. After standing overnight, considerable quantities of mucic acid were precipitated, indicating the presence of galactose or a galactose derivative.

(iii) To the third portion alcohol was added until a whitish precipitate appeared; this was filtered off and dissolved in 25 % nitric acid. On treating this as in (ii) above, mucic acid was again precipitated. The white precipitate was therefore most probably the barium salt of galacturonic acid; this salt is soluble in water and would be formed when excess of barium carbonate was added to the hydrolysis liquid. Further critical examination was not possible owing to the small quantity of material available. Having regard to the nature of pectin it is impossible to conceive that the uronic acid present could be glycuronic acid; moreover, this acid yields a characteristic osazone, which was not detected. The presence of a uronic acid was amply confirmed by the results of quantitative analysis which will be discussed later. The two substances, galactose and galacturonic acid were the only ones detectable in the hydrolysis liquid. No trace of pentose was found, an observation which also was borne out by the results of analysis.

The progress of hydrolysis was followed under standardised conditions, samples being taken from the mixture at definite intervals and reducing sugars being determined by the iodimetric method of Baker and Hulton [1920]. The results in the following table are given in terms of the iodine equivalent to 100 g. of the ash-free product, and recalculated to galactose by the use of the factor 0·73.

Table I. *Rate of hydrolysis of the Fenton product.*

Time in hours	Iodine equivalent to 100 g. g.	Calculated as galactose g.
0	15·17	10·08
0·5	25·67	18·88
1	31·50	23·00
2	37·30	27·20
4	43·01	31·45
6	45·90	33·58
8	49·40	36·10
10	51·20	37·40

It will be seen from Table I that the substance gives slight reduction before hydrolysis and that hydrolysis with sulphuric acid of the concentration employed—1 %—is slow. The substance is therefore comparatively stable and the linkages within the molecule are strong.

The optical rotation of the samples was determined in some cases and the products were found to be strongly dextro-rotatory. Typical results were as follows:

$$\text{Preparation (i)}\ [\alpha]_D^{20^\circ} = +162{\cdot}9^\circ.$$

$$\text{Preparation (ii)}\ [\alpha]_D^{20^\circ} = +161{\cdot}0^\circ.$$

Pectin itself has a specific rotation of the order of + 231°.

Quantitative analysis. In addition to the determination of ash, estimations of total furfuraldehyde by the method of Tollens were carried out, and the yield of carbon dioxide obtained on hydrolysis with 12 % hydrochloric acid was also determined by the method of Nanji, Paton and Ling [1925].

The content of uronic acid anhydride is obtained by multiplying the yield of carbon dioxide by 4 and is shown in the fourth column of Table II. It is also known that the uronic acid anhydrides yield approximately 16·66 % of their weight of furfuraldehyde and consequently the theoretical amount of furfuraldehyde given by the uronic acid groups in the products can be calculated. This calculated figure appears in column 5 of Table II and it will be seen that within the limits of error this approximates to the actual yield of furfuraldehyde obtained experimentally. Hence it is clear that no pentose units are present and the conclusion previously arrived at, that galactose and galacturonic acid are the only products of hydrolysis, is justified.

Table II.

				Furfuraldehyde	
Product No.	Ash %	Carbon dioxide %	Uronic anhydride %	Calculated %	Found %
1	17·27	15·13	60·52	10·08	10·21
2	26·65	14·93	59·72	9·95	10·00
3	26·55	14·44	57·76	9·61	9·85
4	7·68	13·75	55·00	9·14	8·65
5	13·38	13·41	53·64	8·93	8·84
6	14·27	13·26	53·04	8·83	8·41
7	16·42	12·95	51·80	8·63	8·70
8	30·98	12·48	49·92	8·31	8·34

All results are calculated on an ash-free basis.

The table shows a series of products in which the amounts of galactose and galacturonic acid increase and decrease respectively as the table is descended. The last product approximates very closely to a simple unit corresponding to galactose-monogalacturonic acid (uronic acid anhydride content, 49·4 %) and the first products approach the figure—66·16 % uronic acid anhydride—required by a substance whose simplest unit would be galactose-digalacturonic acid. It cannot be definitely stated, however, that these products represent mixtures of these two acids in polymerised form, since there might equally well be present acids whose simple units contain three or four galacturonic acid residues combined with galactose. Again, by an extension of the series in the opposite direction it may be possible to obtain mixtures containing such units as digalactose-monogalacturonic acid. Until further research has been carried out it is only possible to say that the products of oxidation of pectin are mixtures which yield on hydrolysis galactose and galacturonic acid in varying proportions, which depend on the exact conditions of the reaction. These mixtures probably represent stages in the reaction, and were the latter more susceptible to accurate control there seems no reason, in theory, why one or other of the constituents of the mixture should not solely be obtained, as, in fact, appears to be the case with sample No. 8.

DISCUSSION.

The oxidation of pectin by means of Fenton's reagent thus follows a wholly unexpected course. There is no production of the hexo-pentosan which theoretical considerations led us to expect. Nevertheless, the conversion of pectin to a mixture of substances with a strong generic resemblance to the hemicelluloses may be said to have been achieved. The two hemicelluloses of beechwood isolated by O'Dwyer [1926] yielded on hydrolysis arabinose and galacturonic acid, and xylose and glycuronic acid respectively. Norris and Preece [1930] have recently described hemicelluloses of wheat bran which yield in one case two sugars, and in other cases a sugar and a uronic acid on hydrolysis. Norman [1929, 1] has isolated hemicelluloses from cereal straws which yield, similarly, a uronic acid and pentose and hexose sugars. Schorger and Smith [1916] described a substance which they termed ϵ-galactan, a water-soluble and slightly reducing product obtained from the wood of the Western Larch. Careful analysis by them showed that galactose was the only sugar present. The ϵ-galactan, however, yielded 6·18 % of furfuraldehyde. It would seem now that this was undoubtedly due to galacturonic acid and that ϵ-galactan is a substance not unlike those produced by us in the oxidation of pectin. The 6·18 % of furfuraldehyde would be derived from 37·2 % of galacturonic acid anhydride, the remaining 62·8 % presumably consisting of anhydro-galactose. This corresponds very closely with the simple unit represented by digalactose-galacturonic acid. The conviction is therefore growing that the structural hemicelluloses will be found to exist universally as conjugated products, which yield on hydrolysis either two sugars, or, more generally a sugar or sugars, and a uronic acid.

Clearly then, such products as have been obtained by the mild oxidation of pectin fall within the category of hemicelluloses or polyuronides as indicated above. Meanwhile attempts are being made to separate the products obtained into their components.

Norman [1929, 2] has suggested that the formation of substances of the nature of pectin, hemicelluloses and the gums, can be accounted for by the mild oxidation of linked hexosan units. This theory therefore receives some support from the results described above. Oxidation by means of Fenton's reagent is being applied to natural products related to pectin with a view to the publication of further details of the method in the near future.

SUMMARY.

1. Pectin may readily be oxidised by means of Fenton's reagent.

2. The reaction temperature must be carefully controlled, and must not be lower than 30° nor higher than 35°.

3. The products of this exothermic reaction are mixtures, yielding on hydrolysis, galactose and galacturonic acid. They are possibly polymers containing mainly galactose-monogalacturonic acid and galactose-digalacturonic

acid, although the presence of other conjugated acids containing galactose and galacturonic acid is not ruled out.

4. The products resemble in appearance and general properties the structural hemicelluloses, such as those obtained from wheat bran, beech wood and cereal straws.

5. Some support is lent to the view that the hemicelluloses may be formed in nature by the protracted mild oxidation of pectin.

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XLIX. XANTHINE OXIDASE IN THE AVIAN EMBRYO.

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Of the enzymes involved in purine metabolism, it has been shown that in the mammalian embryo xanthine oxidase is one of the last to function and does not appear until in late antenatal or early postnatal life.

Working with the livers of embryo pigs, Jones and Austrian [1907, 1, 2] could not demonstrate its presence in embryos up to 200 mm. in length. Mendel and Mitchell [1907, 1907-8], working with embryos up to 230 mm., came to the same conclusion. These authors found the enzyme to be present in the liver of a suckling pig about 7 weeks old; intermediate stages were not examined, so the exact time of its appearance in the pig's liver cannot be stated. Wells and Corper [1909], working with human foetus, found the enzyme to be present in the liver and viscera at full term, but could not demonstrate its presence at, or before, the 6th month, it presumably being developed between that time and full term. Przylecki and Rogalski [1927], working with glycerol extracts of whole embryos, demonstrated the presence of the enzyme in embryonic chicks on the 7th day.

It seemed of interest to determine with greater precision the period in the development of the avian embryo at which the enzyme makes its appearance in various organs. The earlier studies involved the laborious process of estimating the bases before and after the experimental period. The methylene blue anaerobic technique, as first employed in this connection by Morgan, Stewart and Hopkins [1922] and by Morgan [1926], makes the demonstration of the enzyme an easy and rapid affair.

The organ or tissue is ground up with a measured volume of 2 % sodium fluoride solution, and, in order that any xanthine or hypoxanthine originally present in the extract may be destroyed (by oxidation under the influence of the enzyme), the preparations are allowed to stand for 24 to 48 hours.

The p_H of the solution if below 6·0 being adjusted to this value, the time taken for the reduction of a measured amount of methylene blue is determined, (*a*) in the untreated extract as a control, (*b*) after the addition to the extract of a suitable amount of hypoxanthine.

After treatment as above, the extract by itself reduces very slowly, if at all, whereas, when the oxidase is present, the velocity of reduction is greatly increased by the addition of hypoxanthine. On the other hand, when the enzyme is absent the reduction times of the extract with and without added hypoxanthine are found to agree very closely.

It is clear that the reciprocal of the reduction time gives a not unsatisfactory measure of the relative concentration of the enzyme.

EXPERIMENTAL.

White Leghorn hens' eggs were used for the investigation. The eggs, after the proper time of incubation, were opened, the embryos removed, washed in three changes of distilled water and dried on filter paper. Up to and including the 14th day the whole embryos were used; on the 15th day and after, the dissected organs from several embryos were used.

Preparations were then made from the embryos or organs. Five volumes of 2 % sodium fluoride solution were used for the yolk and yolk sac, and 10 volumes for all other preparations, with the exception of the pancreases, which were ground up with 5 cc. irrespective of their weight.

Table I.

R.T. = Time for complete reduction.

A plus sign in the R.T. columns indicates that the observation ceased before reduction was complete, no difference being then observable between the two tubes compared.

Day of incubation	Nos. of embryos used	Tissue preparation	R.T.						Xanthine oxidase
			Control			Hypoxanthine			
5	10	Whole embryos	11 hrs.		+	11 hrs.		+	–
8	3	,,	16 ,,		+	16 ,,		+	–
9	10	,,	8 ,,		+	8 ,,		+	–
11	3	,,	10 ,,		+	10 ,,		+	–
13	2	,,	8 ,,		+	8 ,,		+	–
15	13	Livers	4 ,,	50 mins.		4 ,,	50 mins.		–
,,	,,	Kidneys	6 ,,		+	0 ,,	37 ,,		+
,,	,,	Other viscera	7 ,,		+	7 ,,		+	–
16	7	Livers	7 ,,	5 ,,		7 ,,	5 ,,		–
,,	,,	Kidneys	7 ,,		+	1 ,,	5 ,,		+
17	13	Livers	5 ,,	7 ,,		5 ,,	10 ,,		–
18	11	,,	5 ,,	5 ,,		5 ,,	5 ,,		–
,,	,,	Kidneys	5 ,,		+	0 ,,	13 ,,		+
19	12	Livers	4 ,,	43 ,,		4 ,,	47 ,,		–
,,	,,	Kidneys	6 ,,		+	0 ,,	10 ,,		+
,,	,,	Pancreas	24 ,,		+	7 ,,		+	Trace
20	5	Livers	3 ,,	12 ,,		3 ,,	12 ,,		–
,,	,,	Kidneys	4 ,,	7 ,,		0 ,,	9 ,,		+
,,	,,	Pancreas	24 ,,		+	11 ,,		+	Trace
,,	,,	Heart	8 ,,		+	8 ,,		+	–
,,	,,	Lung	8 ,,		+	8 ,,		+	–
,,	,,	Muscle	5 ,,		+	5 ,,		+	–
,,	,,	Intestines	6 ,,	32 ,,		6 ,,	32 ,,		–
,,	,,	Gizzard	5 ,,		+	5 ,,		+	–
21	4	Kidneys	6 ,,		+	0 ,,	10 ,,		+
,,	,,	Pancreas	24 ,,		+	10 ,,	0 ,,		+
,,	3	Livers (eggs not chipped)	3 ,,	46 ,,		3 ,,	46 ,,		–
,,	1	,, ,,	5 ,,	38 ,,		5 ,,	38 ,,		–
,,	1	,, ,,	3 ,,	40 ,,		3 ,,	40 ,,		–
,,	5	Livers (chicks' heads through shell)	2 ,,	36 ,,		0 ,,	46 ,,		+
,,	4	,, ,,	2 ,,	39 ,,		0 ,,	38 ,,		+
24 hrs. old chicks	4	Livers	2 ,,	39 ,,		0 ,,	4 ,,		+

Table II.

Day of incubation	Preparation		R.T.				Xanthine oxidase
			Control		Hypoxanthine		
0	Yolk		24 hrs.	+	24 hrs.	+	–
2	,,		24 ,,	+	24 ,,	+	–
3	,,		24 ,,	+	24 ,,	+	–
4	,,		24 ,,	+	24 ,,	+	–
6	,,		24 ,,	+	24 ,,	+	–
,,	,,	sac	24 ,,	+	24 ,,	+	–
7	,,		24 ,,	+	24 ,,	+	–
,,	,,	,,	48 ,,	+	11 ,,		Trace
8	,,		24 ,,	+	24 ,,	+	–
,,	,,	,,	48 ,,	+	11 ,,		Trace
9	,,		24 ,,	+	24 ,,	+	–
,,	,,	,,	4 ,, 3 mins.		1 ,, 15 mins.		+
11	,,		24 ,,	+	24 ,,	+	–
,,	,,	,,	1 ,, 45 ,,		0 ,, 35 ,,		+
13	,,		24 ,,	+	24 ,,	+	–
,,	,,	,,	2 ,, 15 ,,		0 ,, 37 ,,		+
15	,,		24 ,,	+	24 ,,	+	–
,,	,,	,,	2 ,, 35 ,,		1 ,, 13 ,,		+
16	,,	,,	1 ,, 50 ,,		1 ,, 40 ,,		+
18	,,		9 ,, 30 ,,		9 ,, 30 ,,		–
,,	,,	,,	4 ,, 23 ,,		3 ,, 31 ,,		+
19	,,		11 ,,	+	11 ,,	+	–
,,	,,	,,	6 ,, 26 ,,		0 ,, 56 ,,		+
20	,,	,,	4 ,, 35 ,,		1 ,, 7 ,,		+
21	,,		7 ,, 50 ,,		7 ,, 50 ,,		–
,,	,,	,,	6 ,, 35 ,,		3 ,, 30 ,,		+

From 2 to 5 cc. of the preparations were measured into two vacuum tubes. To one was added from 0·2 cc. to 0·5 cc. of a freshly prepared $M/100$ hypoxanthine solution in phosphate buffer p_H 7·6, and to the control the equivalent amount of phosphate buffer. To each was added the same quantity, 0·5 cc. to 1 cc., of $M/100$ methylene blue solution. The tubes were well evacuated, placed in a thermostat with a glass side at 37°, and the reduction time noted (Tables I and II).

DISCUSSION.

It will be seen from the tables that the method used gave results which for the most part were unequivocal. In the majority of the observations, which have been assumed to prove the absence of the oxidase, the R.T. figures for preparations, respectively with and without added hypoxanthine, were practically identical. So frequently was this agreement observed, when the tissues had received the treatment described, that a relatively small increase in the velocity of reduction due to the addition of hypoxanthine (as in the case of the embryo pancreas on and after the 19th day) seems to prove that the oxidase was then present in low concentration.

It will be seen from Table I that the oxidase was present in the kidney from the 15th day and in the pancreas from the 19th. In each case the day mentioned was the earliest upon which the organ in question was examined individually. Before the 15th day whole embryos had to be employed. It is possible that the developing kidney might earlier have contained low concentrations of the enzyme; the organ constituting, however, too small a proportion of the whole embryo for this to be detected. It is certain that the enzyme was absent from all other organs of the embryo examined up to the last day of incubation, when it suddenly developed in the liver.

On the other hand the evidence seems conclusive in showing (Table II) that it may be present in the yolk sac, though not in the yolk, from the 7th day onwards. Since other authors have found that the yolk sac has properties which are not those of an inert membrane it may prove of interest to have shown that a catalyst is associated with its structure.

Przylecki and Rogalski [1927], as mentioned earlier, found some evidence for the existence of the oxidase on the 7th day. It is not clear from their papers whether the material they extracted contained the yolk sac or not.

I have previously [1926] reported a negative result for the pancreas of the fowl but the enzyme is undoubtedly present, although in relatively low concentration, in the White Leghorn embryo and chick's pancreas used in this research. This may be a question of age, or of activity of the gland and will need reinvestigation.

The circumstances of the appearance of the oxidase in the liver constitute the most interesting point revealed by this investigation. So far as its late appearance in this organ is concerned the results are in accordance with what has been found in the mammalian embryo; but my observations indicate that the appearance is, in the case of the fowl, a highly critical phenomenon. I have found consistently that on the last day of the incubation, before the chicken has broken the shell, no evidence of the presence of the enzyme in the liver can be obtained. Yet by the time a hole is made big enough for emergence (representing but a few hours' activity on the part of the young bird) the oxidase is found in the organ in highly effective concentration. There is a further increase during the first day after emergence.

It would seem that any speculations concerning the nature of biological catalysts of this type must take account of the fact that their appearance in a tissue may be of this sudden kind.

SUMMARY.

1. The embryo of the chick has been investigated for the presence of xanthine oxidase. It is present in the kidney on the 15th day and in the pancreas on the 19th day, the earliest days on which the individual organs were tested.

2. It is not present in the liver until the 21st day when its appearance is strikingly sudden.

My thanks are due to Sir F. G. Hopkins for giving me opportunities for making the observations and to Dr Needham for the supply of the embryos employed.

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L. FACTORS AFFECTING THE CATALYTIC ACTION OF COPPER IN THE OXIDATION OF CYSTEINE.

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The catalytic effect of salts of copper and other metals on the oxidation of cysteine was first demonstrated by Mathews and Walker [1909]. The greatest activity was obtained in a neutral solution of cysteine and the least in an acid solution. In spite of this work the reaction was considered for many years to be one of autoxidation in which the cysteine, when under favourable conditions, is oxidised directly by atmospheric oxygen. However, the so-called autoxidation was shown by Warburg and Sakuma [1923] to be due entirely to the presence of heavy metals, and to be to a large extent inhibited by KCN, the effect of which they ascribed to the formation of an inactive compound with the heavy metals present.

Toda [1926] showed that the addition of iron accelerated the reduction of methylene blue by cysteine and that this reaction was inhibited by KCN. Harrison [1927] found that copper was also active in stimulating the anaerobic oxidation of cysteine and thioglycollic acid, and that cyanide was equally effective in the inhibition of the reaction under these conditions as in aerobic oxidation. These observations undoubtedly prove that the oxidation of cysteine in any appreciable amount takes place only in the presence of certain heavy metals.

The catalytic effect of heavy metals on the oxidation of cysteine has been used by Warburg [1927] for the quantitative determination of these elements when present in minute traces. He found that pyrophosphate inhibited the activity of iron and manganese completely, but did not affect the action of copper in the oxidation of cysteine. He could therefore determine quantitatively small amounts of copper in the presence of iron and manganese by following the rate of oxygen uptake with cysteine in a pyrophosphate buffer solution under fixed conditions.

Apart from these studies very little work has been done on the various factors influencing the action of copper in the oxidation of cysteine. Dixon and Tunnicliffe [1923], before the importance of the presence of heavy metals was realised, made a very complete study of the factors affecting the oxidation of cysteine, as well as of glutathione and thioglycollic acid. It is difficult to say, however, whether the oxidation obtained in the case of these sulphydryl compounds was due to the presence of iron, copper, or even other metals. A study of the factors operative when copper alone is responsible for the oxidation is, therefore, important.

The fact that copper may interfere when cysteine is used for the study of oxidase activity was recently demonstrated by Keilin [1930], who used the rate of cysteine oxidation to determine the activity of an oxidase-cytochrome system. When he added $M/100$ pyrophosphate to the mixture of cysteine, cytochrome, and oxidase, in an orthophosphate buffer at p_H 7·3, to determine if any of the activity was due to free iron, which pyrophosphate would inhibit, he found that there was a great acceleration in the oxygen uptake rather than an inhibition. Keilin suggests that this acceleration is due to the action of pyrophosphate on the copper which may be present in the heart muscle preparation used as the source of oxidase. A quantitative determination of copper shows that there is a sufficient amount of this element present to account for the increased oxygen uptake observed.

Dixon and Elliott [1929] found that the addition of small concentrations of pyrophosphate caused a slight acceleration in the respiration of liver in buffer solutions. This may also be due to the action of pyrophosphate on the copper in the liver, because no acceleration was observed in the case of muscle which is much lower in copper. Of course in the direct measurements on tissues the action of pyrophosphate is complicated by the presence of iron.

The following experiments are recorded to explain this phenomenon as well as to demonstrate other factors which affect the catalytic properties of copper.

EXPERIMENTAL.

All the measurements were made aerobically and the rate of oxygen uptake was measured in Barcroft differential manometers. The right hand flask of the apparatus contained in most cases 3 cc. of buffer solution including the cysteine and copper additions. Equivalent amounts of buffer solution were always placed in the left hand flask together with similar amounts of muscle tissue, when measurements were made with muscle as a source of copper. 0·3 cc. of 10 % NaOH was placed in a small tube fused to the bottom of each flask. The apparatus was shaken in a water-bath at either 20° or 37°. Three to five minutes were allowed for equilibration, then the stopcocks closed, and readings taken at definite intervals. Extreme care was taken to free the flasks from any traces of copper by washing them with cleaning solution, tap water, HCl, tap water, and finally rinsing them several times with redistilled water.

All solutions and reagents were especially prepared to eliminate traces of copper. The water used was redistilled from an all glass apparatus.

Cysteine hydrochloride. Pure cysteine hydrochloride was prepared by recrystallising twice from concentrated redistilled HCl[1]. The crude cysteine hydrochloride was dissolved in the least amount of hot HCl necessary for complete solution, the solution cooled on ice, and the crystals filtered. After this process had been twice repeated, the crystals were dried in a vacuum desiccator over calcium chloride and sodium hydroxide. The purity of this preparation is demonstrated by the exceedingly low oxygen uptake recorded when it is subjected to optimum conditions of oxidation. A considerable loss of cysteine is encountered in this method, but the quality of the product justifies the loss. The cysteine hydrochloride was brought to the proper p_H with pure NaOH before being added to the mixture.

Pyrophosphate. A concentrated solution of sodium pyrophosphate was made, filtered, adjusted to approximately p_H 8·0, at which p_H copper pyrophosphate is soluble, and cooled with ice to allow the salt to crystallise. The salt was filtered off and dried at 100°. $M/5$ and $M/10$ solutions of sodium pyrophosphate were made by dissolving the theoretical amount in water and adjusting the p_H, in most cases to 7·6, with N HCl.

Sodium hydroxide. Sodium hydroxide free from iron and copper was prepared by first allowing a 40 % solution to stand for several days and decanting the clear solution from any precipitate formed. A 0·2 M solution was then made and treated in the same manner.

Phosphate buffer. This was made according to Clark and Lubs from acid potassium phosphate and sodium hydroxide. The 0·2 M solution of acid phosphate was allowed to stand several days to precipitate any iron or copper. After adding the proper amount of NaOH to give the desired p_H, the solution was diluted to $M/15$ or $M/30$ concentration.

Borate buffer. This was made from $M/20$ borax and $M/5$ boric acid according to the table of Palitzsch. These salts were used directly without further purification, but blank determinations showed that they contained very little copper.

Copper solution. Made by diluting a standard solution of copper sulphate containing 0·1 mg. Cu per 1 cc. to give one which contained 1×10^{-4} mg. Cu per 0·1 cc.

Blank determinations were made first in order to determine the purity of the cysteine hydrochloride and the buffer solutions used. The oxygen uptake was measured with cysteine in $M/15$ orthophosphate buffer p_H 7·6, and also with cysteine in the phosphate buffer containing $M/100$ pyrophosphate. Similar measurements were made with Kahlbaum's cysteine (nach Prof. Warburg) to compare the degree of purity of the two samples. The results in Table I show that the cysteine prepared by recrystallisation contained less metal than the Kahlbaum preparation. The addition of pyrophosphate increased the oxygen uptake in both cases very slightly. This increase was not due entirely to any possible metals in the pyrophosphate but to a large extent to the accelerating action of the pyrophosphate on the slight amount of copper in the solutions used. When larger amounts of pyrophosphate were used, the oxygen uptake was no higher. One of the manometers in each series was generally used for a blank run and the oxygen uptake due to cysteine alone was always deducted in calculations involving an absolute amount of copper, but not in the results where only relative values were required. Whenever a relatively large oxygen uptake was observed in the blank, the contaminated solution was replaced by a new one. It is very difficult to prevent contamination when dealing with such minute quantities, therefore the solutions used for each series were never pipetted from the stock solution but from aliquots.

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[1] I am indebted to Dr M. Dixon for suggesting this method of purification.

Table I. *Oxygen consumption by pure cysteine.*

	mm.³ oxygen absorbed per hour	
Cysteine	$M/15$ buffer	Buffer plus $M/100$ $Na_4P_2O_7$
No. 1	1·2	5·0
Kahlbaum	5·4	12·5

Effect of pyrophosphate.

Results demonstrating the accelerating action of pyrophosphate on a heart muscle preparation of oxidase are given in Fig. 1. The concentrated heart muscle suspension was prepared by Dr Keilin according to his usual procedure [Keilin, 1929]. 10 cc. of this strong suspension was diluted to 50 cc. with $M/15$ phosphate buffer p_H 7·6 and 1·5 cc. of the diluted mixture used in the Barcroft flask, or an amount equivalent to 0·3 cc. of the strong mixture.

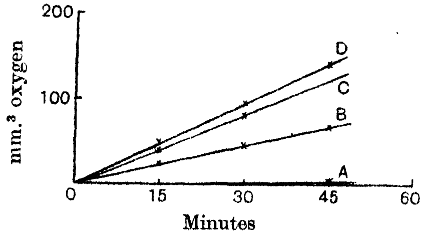

Fig. 1. Effect of pyrophosphate on the activity of copper in muscle tissue. 8 mg. cysteine in 3 cc. phosphate buffer p_H 7·6. A, pyrophosphate alone. B, muscle alone. C, muscle + pyrophosphate. D, 2×10^{-4} mg. copper + pyrophosphate.

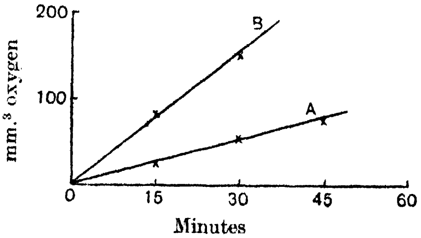

Fig. 2. Effect of pyrophosphate on the activity of copper solutions. 8 mg. cysteine in 3 cc. phosphate buffer p_H 7·6. A, copper alone 4×10^{-4} mg. Cu. B, same amount of copper + pyrophosphate.

Each flask contained a final volume of 3 cc. of orthophosphate buffer together with the various additions. *A*, contained the buffer plus $M/100$ pyrophosphate; *B*, the buffer plus muscle alone; *C*, the buffer plus muscle and pyrophosphate; *D*, the buffer plus 2×10^{-4} mg. Cu with pyrophosphate. The rate of oxidation with the same amount of muscle is twice as rapid in the presence of pyrophosphate as in the buffer solution alone. The rate obtained with 0·3 cc. strong muscle suspension with pyrophosphate is about the same as that with 2×10^{-4} mg. Cu under the same conditions. Therefore, 0·3 cc.

contains about 2 × 10^{-4} mg. Cu. A determination of Cu in 75 cc. of the muscle suspension by the method of Elvehjem and Lindow [1929] gave a copper content of 0·0006 mg. Cu per 0·3 cc. Only about one-third of the copper present is therefore available for catalytic action. This is not surprising because undoubtedly a large proportion of the copper present comes from the sand used during the preparation (the muscle pulp contains considerably more copper than that attributed to the heart muscle itself), and all the copper is made soluble only by ignition and extraction with strong HCl. Several other determinations gave results of similar order.

Pyrophosphate has the same accelerating action whether the source of copper is muscle tissue suspension or soluble copper sulphate. In the presence of 4 × 10^{-4} mg. Cu the rate of oxidation is almost three times as rapid when pyrophosphate is added as when the phosphate buffer is used alone. Curves demonstrating the differences in the rate of oxidation are given in Fig. 2.

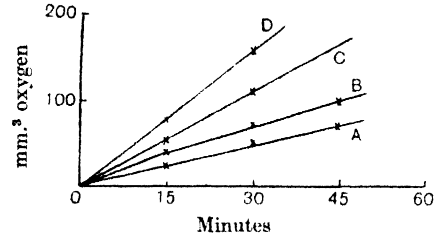

Fig. 3. Action of H_2S in presence and absence of pyrophosphate. 8 mg. cysteine and 4 × 10^{-4} mg. copper in 3 cc. phosphate buffer p_H 7·6. A, copper alone. B, copper + H_2S. C, copper, H_2S and pyrophosphate. D, copper and pyrophosphate.

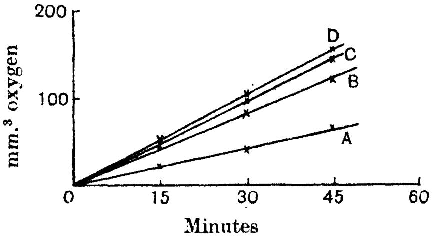

Fig. 4. Effect of phosphate buffer concentration on the activity of copper. 8 mg. cysteine and 4 × 10^{-4} mg. copper in 3 cc. phosphate buffer. A, $M/15$ buffer. B, $M/20$. C, $M/30$. D, $M/60$.

This difference in activity is more readily explained by the inhibitory effect of the phosphate buffer than by the accelerating action of the pyrophosphate. The actual inhibitory effect of $M/15$ orthophosphate buffer p_H 7·6 was noticed when the influence of H_2S on the catalysis was studied. The addition of sulphide decreased the activity of the copper in the presence of pyrophosphate, but a decided increase was observed in the case of the buffer solution alone. The oxygen uptake of the different solutions is given in Fig. 3. The inhibitory action of sulphides agrees with the earlier results obtained with H_2S and is probably due to the formation of copper sulphide, but the accelerating effect must be explained in another way. Since the H_2S was added as an aqueous solution of Na_2S (0·3 cc. $M/100$ Na_2S per 3 cc.), the increased action is probably due to the dilution of the buffer solution. The copper becomes more soluble or more available in the more dilute solution. Fig. 4 shows that dilution actually hastens the rate of oxygen consumption. Decided increases in the rate are noted as the buffer is diluted from $M/15$ to $M/20$ and $M/30$, but further dilution causes very little change in the reaction.

Effect of hydrogen ion concentration.

These facts suggest that the solubility of the copper in the buffer solution is the limiting factor. If a weak solution of copper sulphate is added to the phosphate buffer at p_H 7·6 a precipitate of copper phosphate is formed immediately, but if the same amount of copper is added to a pyrophosphate solution at the same p_H no precipitate is formed. Variations in the p_H undoubtedly change the solubility of the copper and thus should bring about certain differences in the rate of oxygen consumption. Results of the measurements made at different H ion concentrations for $M/15$ and $M/30$ phosphate, and for $M/15$ phosphate plus pyrophosphate are given in Fig. 5. In the case of $M/15$ phosphate variations in the p_H have no significant effect on the oxidation rate, because at this concentration the added copper is changed to insoluble copper phosphate at all degrees of acidity. However, when the lower concentration of phosphate was used a maximum activity was noted at p_H 7·6.

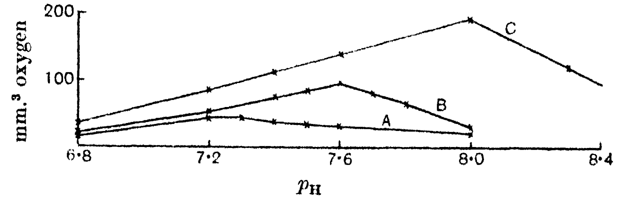

Fig. 5. The effect of p_H on the activity of copper in different buffer solutions. Oxygen uptake at the end of 30 minutes. 8 mg. cysteine and 4 × 10^{-4} mg. copper. A, $M/15$ phosphate buffer. B, $M/30$ phosphate buffer. C, $M/15$ phosphate buffer + pyrophosphate.

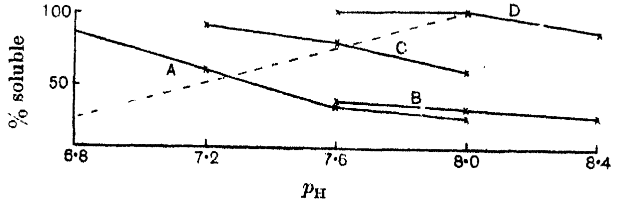

Fig. 6. The solubility of copper in different buffer solutions. 25 cc. buffer solution with 0·1 mg. copper. A, $M/15$ phosphate buffer. B, borate buffer. C, $M/30$ phosphate buffer. D, $M/15$ phosphate buffer + pyrophosphate.

The copper is most soluble at this H ion concentration and decreases in solubility as the solution becomes more alkaline. The presence of pyrophosphate gives some increase in activity over $M/30$ buffer at p_H 7·6, but a maximum is obtained at p_H 8·0 with a decrease as the p_H is further increased. Since copper pyrophosphate is soluble in more alkaline solutions than copper phosphate, it is possible for the maximum activity to occur at a higher p_H. Borate buffer solutions were used for p_H values above 8·0. The oxygen uptake with pyrophosphate in phosphate buffer and in borate buffer at p_H 8·0 was measured under the same conditions. Identical results were obtained showing that the type of buffer in which the pyrophosphate acts has no influence on the rate of reaction.

In order to demonstrate that the suggested changes in solubility occur, the solubility of a definite amount of copper at the various H ion concentrations was determined. 0·1 mg. of Cu was added to 25 cc. of the buffer solution adjusted to the proper p_H, allowed to stand 2 hours, filtered, and the amount of copper remaining in the filtrate determined. 3 cc. of $M/10$ $Na_4P_2O_7$ were added to the solution containing the pyrophosphate. The percentage of soluble copper is plotted against p_H in Fig. 6.

Although it is difficult to compare the solubility curves with those obtained by oxygen consumption, because the former were measured when 0·1 mg. Cu was used with 25 cc. of buffer, and the latter when 0·0004 mg. was used with 3 cc. of buffer, it is readily seen that the curves are similar for the phosphate buffer on the alkaline side of p_H 7·6 and for the pyrophosphate buffer on the alkaline side of p_H 8·0. The retarding action of increased alkalinity in either of the buffers is due to the formation of insoluble copper salts. The slope of the oxygen uptake curves on the acid side of p_H 7·6 and 8·0 cannot be explained by the solubility of the copper in relation to the buffer, because the solubility increases with the increased acidity in all cases.

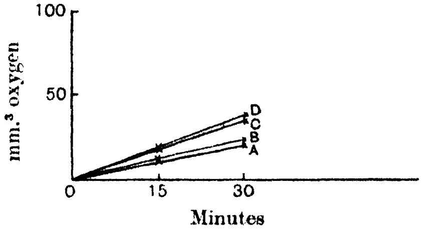

Fig. 7. Comparison of copper activity with and without buffer. 8 mg. cysteine and 2×10^{-4} mg. copper. A, cysteine in buffer p_H 6·8. B, cysteine alone p_H 6·8. C, cysteine in buffer p_H 7·6. D, cysteine alone p_H 7·6.

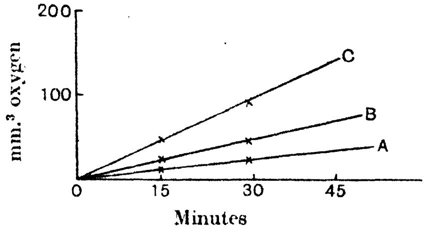

Fig. 8. Effect of copper concentration on the rate of oxidation. 8 mg. cysteine in $M/15$ phosphate buffer p_H 8·0 with $M/100$ pyrophosphate. A, $0{\cdot}5 \times 10^{-4}$ mg. Cu. B, 1×10^{-4} mg. Cu. C, 2×10^{-4} mg. Cu.

Because the solubility determinations were made on the buffer solutions alone without the addition of cysteine, the decreasing activity with increased acidity may be attributed to the action of cysteine. The cysteine is undoubtedly the active factor because the slopes of the curves are identical irrespective of the buffer used. The same oxygen consumption is obtained whether the oxidation is carried out in buffer solution, or in water solution where the cysteine itself is used as the buffer. The results of these experiments are given in Fig. 7. If a line be drawn in Fig. 6 between p_H 6·8 and 8·0 which has the same slope as the curve for oxygen uptake in Fig. 5, it will intersect the solubility curves at p_H 7·2 for $M/15$ phosphate, at p_H 7·6 for $M/30$ phosphate, and at p_H 8·0 for pyrophosphate. The optimum oxygen uptake for the different solutions is actually observed at these p_H.

The retarding effect of cysteine in more acid solutions cannot be explained by the insolubility of a copper-cysteine complex, for, although copper forms a precipitate with cysteine at p_H 6·0 and below, no precipitate is formed between p_H 6·0 and 8·0 even though 1 to 2 mg. Cu are added to 10 mg. cysteine. However, an inactive copper-cysteine complex may be more readily formed in acid solutions than in neutral or alkaline solutions. Further reference to this complex will be made when the results obtained at 37° are presented.

Since the rate of oxidation of cysteine is greatest with pyrophosphate in buffer solution at p_H 8·0, the buffer and the cysteine are exerting the least retarding effect, and, therefore, this H ion concentration should give the proper conditions for the quantitative determination of copper. The curves in Fig. 8 show that the rate of oxidation of 8 mg. cysteine in 3 cc. $M/15$ phosphate or borate buffer at p_H 8·0 and containing $M/100$ pyrophosphate is directly proportional to the copper present for amounts below 4×10^{-4} mg. Cu. This procedure is quite similar to that used by Warburg [1927] for the determination of copper except that a low concentration of pyrophosphate in a rather dilute buffer is used in place of a strong pyrophosphate buffer alone. This method allows the use of more dilute buffer solutions because the orthophosphate forms a more efficient buffer than pyrophosphate.

Interfering action of iron.

The above results were obtained when copper was the only heavy metal present, but the interfering effect of other metals must also be considered. Addition of iron in amounts equivalent to the copper, or to 2 to 3 times the amount of copper, does not affect the oxygen uptake materially as is shown in Table II. The iron was added as ferric chloride which was prepared free from traces of copper. The failure of small amounts of iron to interfere is, of course, in agreement with Warburg's findings. However, if larger amounts of iron (0·02 mg., approximately equivalent to the amount present in 0·5 cc. whole blood) are used, a very large inhibition is observed. The amount of inhibition decreases as the amount of pyrophosphate used is increased but the normal rate is not reached even if $M/5$ pyrophosphate, suggested by

Table II. *Effect of iron on the catalytic action of copper in the presence of pyrophosphate.*

Conditions of cysteine oxidation					mm.^{3} oxygen per ½ hour		
Phosphate buffer	Pyrophosphate	Copper mg. $\times 10^{-4}$	Iron mg. $\times 10^{-4}$	Temp. ° C.	Copper	Copper + iron	Recovery %
$M/15$ p_H 8·0	$M/100$	4	4	20	142·0	136·0	96·0
,, ,,	,,	2	400	,,	86·7	23·0	26·0
,, ,,	$M/30$	1	,,	,,	50·7	30·1	59·0
,, p_H 7·6	$M/100$	1	,,	,,	35·4	16·0	45·0
—	$M/5$ p_H 8·0	1	200	,,	58·3	48·0	83·0
$M/15$ p_H 7·8	$M/10$	1	,,	,,	46·7	34·8	75·0
,, p_H 7·6	$M/10$	1	,,	37	63·3	54·3	86·0
,, ,,	$M/8$	1	,,	,,	72·9	70·8	97·0

Warburg, is added. Warburg's figures show the same effect; when he added 0·02 mg. Fe together with 2×10^{-4} mg. Cu to $M/5$ pyrophosphate at p_H 7·6 only an 80 % recovery was obtained.

Figures given in Table II show that a complete recovery of copper is never obtained, although the procedure is modified as to p_H, concentration of pyrophosphate, etc., when the reaction is carried out at 20°. When the oxidation takes place at 37° with rather high concentrations of pyrophosphate, the rate in the presence of relatively large amounts of iron is practically identical with that obtained with copper alone. The retarding effect of iron in the presence of pyrophosphate is most probably due to the formation of insoluble ferric pyrophosphate which inactivates some of the copper. There are two reasons for this view. Firstly, it is known that iron pyrophosphate is soluble in alkaline solutions only in the presence of excess pyrophosphate, and the addition of more pyrophosphate increases the recovery of copper; secondly, ferric pyrophosphate is more soluble at 37° than at 20° and the recovery of copper is better at 37°. The latter fact can be demonstrated by taking two solutions of pyrophosphate at p_H 7·6 and adding a few drops of dilute iron solution to one at 37° and the other at 20°, the precipitate formed, upon the addition of iron, disappears most rapidly in the first solution.

The complete recovery of copper in the presence of iron at 37° is further demonstrated when the copper content of a solution from the ash of liver is determined by this method and by the method of Elvehjem and Lindow [1929]. 140 mg. Cu was found by the first method and 125 mg. Cu by the latter. These results agree exceedingly well when one considers that the copper content of the sample analysed was of the order of 1×10^{-4} mg. in one case and 0·1 mg. in the other. They further show that there are no other elements in the ash of liver which accelerate the oxidation of the cysteine in the presence of pyrophosphate.

Effect of temperature.

It is interesting to note that the optimum p_H at the higher temperature (37°) is no longer at 8·0 but nearer 7·0. This is not merely a temperature effect, because in a number of carefully controlled experiments the oxygen consumed per 30 minutes in the presence of 1×10^{-4} mg. Cu at p_H 7·6 was found to be 32–36 mm.^{3} at 20° and 50–54 at 37°, but 80–85 for p_H 7·0 at the latter temperature. The relation of p_H to the oxygen uptake at 37° in the presence of pyrophosphate is shown in Fig. 9. An optimum is observed at p_H 7·0 with a rapid falling off as p_H 6·6 is reached. The pyrophosphate curve for 20° is replotted in this figure to show the relationship of the two.

The fact that the inhibiting action of cysteine is decreased in acid solution at 37° allows one to make several other observations on the activity of copper. Firstly, curve C in Fig. 9 shows that even in the less alkaline solution pyrophosphate is still needed for the optimum activity of the copper. The uptake, however, is higher with phosphate alone at 37° than at 20° and the p_H curve is similar to the solubility curve, which further proves that the retarding effect of the more acid solution is due to the inactive copper-cysteine complex formed.

Greater activity of the copper is not obtained by carrying out the reaction in water, with cysteine instead of phosphate as a buffer. Undoubtedly in the absence of phosphate the copper is precipitated as hydroxide instead of copper phosphate, because it can be shown that a precipitate is formed when a dilute solution of copper sulphate is brought to p_H 7·0. Fig. 10 gives the results obtained when the oxygen consumption was measured in a watery solution, phosphate solution, or a phosphate plus pyrophosphate solution. The activity without the pyrophosphate is similar whether in watery or phosphate solution, but considerably lower than when pyrophosphate is added. Results of the same order were obtained when heart muscle preparation was used as the source of copper.

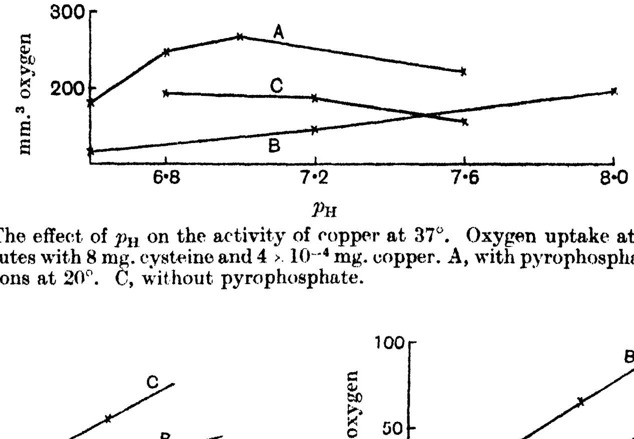

Fig. 9. The effect of p_H on the activity of copper at 37°. Oxygen uptake at the end of 30 minutes with 8 mg. cysteine and 4 × 10^{-4} mg. copper. A, with pyrophosphate. B, same conditions at 20°. C, without pyrophosphate.

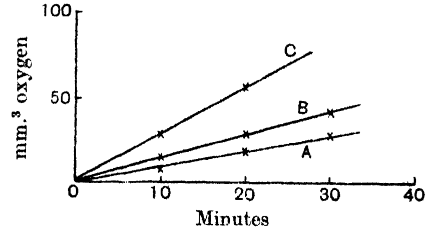

Fig. 10. The influence of buffer on the activity of copper at 37°. 8 mg. cysteine and 1 × 10^{-4} mg. copper at p_H 7·0. A, without buffer. B, phosphate buffer. C, buffer + pyrophosphate.

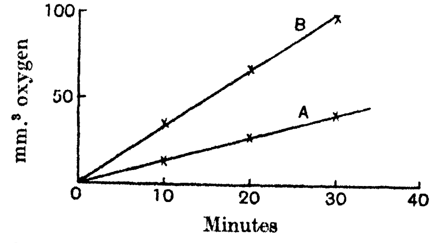

Fig. 11. The effects of H_2S on the activity of copper. 8 mg. cysteine and 1 × 10^{-4} mg. copper in $M/15$ phosphate p_H 7·0 with pyrophosphate. A, copper + $M/1000$ H_2S. B, copper alone.

Secondly, by working at the higher temperature a high rate of oxygen uptake is obtained over a relatively wide p_H range, which allows a study of the effect of p_H on inhibition by H_2S. A typical result obtained by the addition of H_2S at p_H 7·0 is given in Fig. 11. The percentage inhibition at this p_H is 60, but the amount decreases at higher p_H values and 40 to 50 % is obtained at p_H 7·6. At p_H 8·0 very little inhibition is observed and in some cases an actual increase results by the addition of H_2S, possibly due to the oxidation of the sulphide.

Krebs [1929] found that copper as well as other heavy metals catalyses the oxidation of sulphides. It is difficult, however, to compare his results with these experiments because he worked with very high concentrations of sulphide (0·5 *M*) and used the sulphide without buffer. The solution was undoubtedly quite alkaline, because when similar reactions were carried out in *N* HCl the rate of oxidation was very slow.

Further observations on the activity of copper, which need merely be recorded without including the complete experimental results because they only confirm previous work, are the following. The activity is entirely unaffected by the presence of carbon monoxide but is inhibited to at least 95 % of the total by *M*/1000 KCN. *M*/100 sodium pyrophosphate is the optimum concentration for the activity of copper in absence of iron, but *M*/5 may be used without any interference. However, when the concentration is decreased to *M*/1000 a definite falling off in the activity is observed and considerably less than 50 % of the total activity is obtained with *M*/3000 pyrophosphate.

Discussion.

Although the work presented in this paper deals entirely with the quantitative study of the catalytic action of copper in the oxidation of cysteine, the results have undoubtedly a broader significance. It would be entirely erroneous to say that the facts observed in this particular system can be applied directly to the action of copper on other compounds, but the tremendous activity of this element in the oxidation of cysteine under optimum conditions may explain how the presence or absence of minute traces of it can bring about profound changes in plant and animal phenomena.

The optimum conditions observed in the case of cysteine may be totally different from those for the optimum oxidation of other compounds, even such as thioglycollic acid and glutathione which closely resemble cysteine. Independent studies need to be made for each of the materials activated by copper.

All the factors shown to be necessary for the optimum activity of copper are ordinarily present in most plants and animals. Copper is universally distributed in small amounts in all plants and animals thus far analysed [Lindow, Elvehjem, and Peterson, 1929]. The optimum p_H at 37° is between 7·0 and 7·6 which is the hydrogen ion concentration associated with a large portion of living matter. Copper is more active in the presence of pyrophosphate and Lohmann [1928] has found pyrophosphate in the muscle of certain animals and in yeast, while Eggleton and Eggleton [1929] have reported its presence in blood.

Summary.

1. The oxygen uptake of a system of cysteine and heart muscle preparation in orthophosphate buffer p_H 7·6 is increased two or threefold by the addition of pyrophosphate.

2. The same acceleration is obtained when the muscle preparation is replaced by a minute amount of copper salts. The increased rate of oxidation in the presence of pyrophosphate is due to the formation of copper pyrophosphate, which is more soluble at p_H 7·6 than copper phosphate and therefore more catalytically active.

3. Curves are presented to show the rate of cysteine oxidation by copper in phosphate buffers of different concentrations, and in a phosphate buffer plus pyrophosphate at varying hydrogen ion concentrations.

4. A study of the solubility of copper in different buffer solutions shows the decreased activity with increased p_H to be due to the formation of insoluble copper phosphates. The decrease of activity, as the p_H becomes more acid, can only be explained by the formation of a soluble inactive copper-cysteine complex.

5. The optimum activity of copper in phosphate buffers containing pyrophosphate occurs at p_H 8·0 at 20°. At this hydrogen ion concentration the rate of oxidation is directly proportional to the copper present, providing the copper is not in excess of 4×10^{-4} mg. Cu, and thus provides a method for the quantitative estimation of minute amounts of copper.

6. Relatively large amounts of iron inhibit the activity of copper in the presence of pyrophosphate. This retarding effect can be eliminated by using larger concentrations of pyrophosphate and by carrying out the reaction at 37°. At 37° the optimum activity is observed at p_H 7·0, and this is undoubtedly due to the fact that the copper-cysteine complex is less readily formed at the higher temperature.

7. The inhibiting action of cysteine at p_H 7·0 disappears at 37°, but the copper is sufficiently insoluble in buffer or watery solutions even at this p_H to prevent its complete activity in the absence of pyrophosphate.

8. $M/1000$ H_2S inhibits the activity of copper in phosphate buffers containing pyrophosphate to the extent of 60 to 70 % at p_H 7·0, but the degree of inhibition is much less at higher p_H values.

I wish to thank Sir F. G. Hopkins and Dr D. Keilin for their interest and advice.

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LI. THE ACTION OF SALTS ON FUMARASE. I.

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Fumarase is the name given by Batelli and Stern [1911] to the enzyme in animal tissues capable of converting fumaric acid into malic acid. It was shown by Einbeck [1919] that the reaction reached equilibrium when three-quarters of the fumaric acid had been changed, and by Dakin [1922] that the malic acid so formed was entirely in the laevo-form. Clutterbuck [1927, 1928] followed polarimetrically the production of *l*-malic acid from fumaric acid under the influence of the muscle and liver enzyme, and found that the reaction followed a linear course until the equilibrium state was almost reached. He also showed that the velocity of the action was influenced by salts, phosphates at p_H 7·2 producing a marked activation, and chlorides, bromides, fluorides and nitrates an inhibition.

Quastel and Whetham [1924] reported that fumarase was present in very small amount in *B. coli communis*. It was shown by Woolf [1929] that the fumarase activity, in the experiments of these workers, was masked by a side reaction, which could be inhibited by 2 or 4 % propyl alcohol; in presence of this inhibitor *B. coli communis* displayed great fumarase activity. Woolf reported that with the bacterial enzyme reaction curves were obtained similar to those given by Clutterbuck for the animal preparations, the velocity of conversion of fumaric acid remaining constant for the greater part of the reaction. He also showed that phosphates had an accelerating effect on the enzyme at p_H 7·4.

Although there are many known cases of enzyme activity being accelerated or retarded by salts, there are hardly any enzymes for which such effects have been studied in detail. Such an investigation has been carried out by Myrbäck [1926] on salivary amylase. As is well known, sodium chloride has a marked accelerating action on this enzyme. Myrbäck studied the effect of this salt, as well as the action of bromides, iodides, nitrates and chlorates, all of which activate the enzyme. He showed that at any given p_H increasing concentrations of the salt gave first of all an increasing activation, until a maximum effect was obtained which did not change with increase of salt. The curve relating salt concentration to increase in enzyme velocity seemed to be a rectangular hyperbola, or "Michaelis curve," suggesting that the salt, or one of its ions, combined with the enzyme, the amylase-salt complex being more active than the free amylase. The presence of the salt did not markedly change the affinity of the enzyme for its substrate, so that the salt combined with the enzyme at some group other than that concerned in binding the substrate. On studying the effect of p_H on the activity of the free enzyme and of the various enzyme-salt compounds, he found that the latter were active over a wider p_H range than the salt-free preparation. The p_H-activity curves were related together like the dissociation-residue curves of an ampholyte, in which the p_K of the $H^{\cdot}$ and the OH' ions was shifted to various extents by the added salts. He concluded that the enzyme was an ampholyte, which was catalytically active only in its undissociated or isoelectric form. Combination with a salt altered the degree of dissociation of the enzyme, and hence the proportion of the enzyme in the active form at any given p_H.

It seemed desirable to study the effects of salts on fumarase, and the present paper gives the results of the first part of this investigation. The salts used were the phosphate, citrate and sulphate of sodium. The sodium was always present in large concentration, being used to neutralise the substrate as well as the added acid, and its influence (if any) on the reaction velocity may be taken as constant in all the experiments described. The effects observed can therefore be attributed to the added anions. The phenomena fit in exactly with the idea that the enzyme is an ampholyte acting only in the isoelectric form, and that it combines with anions, the fumarase-anion complex being an ampholyte with different p_K values, as suggested by Myrbäck for salivary amylase.

EXPERIMENTAL.

The enzyme preparation used throughout this work was a suspension of *B. coli communis*. The organism was grown on the surface of tryptic broth agar in Petri dishes for 2 days. The growth was taken up in distilled water and thoroughly washed by centrifuging several times. It was then again suspended in water, aerated for a few hours and shaken mechanically to ensure an even suspension. This stock preparation of the "resting" organism was stored in the refrigerator. It was generally used within a fortnight of preparation, though it retained its activity unimpaired for several weeks at least. Immediately before use the organism was again washed several times with distilled water to remove any trace of salts produced by autolysis, then made up to a suitable strength with distilled water and shaken in the machine. In this way an enzyme preparation could be obtained free from all salts or other soluble materials. The actual experiments rarely lasted more than 18 hours, and during this period there was no significant enzyme destruction or bacterial disintegration, since the reaction velocity remained constant even for a much longer period, and the enzyme remained associated with the cells, the supernatant fluid after centrifuging being entirely inactive. The effects observed, therefore, may be regarded as given by the enzyme *in vivo*.

The long initial linear portion of the curve, when the equilibrium is approached from the fumaric acid side, made it possible to take the initial velocity as proportional to the malic acid produced in a given time, even when the reaction had progressed a considerable distance. It was thus possible to obtain large polarimetric readings, and thereby to obtain a very accurate measurement of the reaction velocity. We have always obtained this initial linearity in the experiments reported below. The enzyme was therefore always working at full saturation with its substrate, so that the effects could not be due to a change in the affinity of the fumarase for fumaric acid.

For each experiment a series of reaction mixtures was made up, in which one factor was continuously varied. These series were of two kinds—those in which the effect of an increasing concentration of a salt was studied at a constant p_H, and those in which the salt concentration was kept constant, but the p_H was varied. In all cases the initial concentration of fumaric acid was $M/10$, the inhibitor being 4 % propyl alcohol. The reaction mixtures were placed in stoppered flasks and incubated at 37°. At suitable intervals 10 cc. samples were taken for the polarimetric estimation of malic acid as the molybdate compound by the method of Auerbach and Kruger [1923]. The procedure described by Woolf [1929] was followed, save that, as 10 cc. samples were taken, the addition of 5 cc. of water was not required. Also, since *l*-malic acid was the only optically active compound present, there was no need to take the control reading in trichloroacetic acid.

For a series at a constant p_H, the various constituents were made up to suitable concentrations separately, and brought to the required p_H with NaOH. The p_H of the bacterial suspension was also adjusted. The required quantities were then mixed and made up to volume with distilled water, and the p_H of the mixture was checked. For a series with varying p_H, two solutions were made up, one more acid and the other more alkaline than the extremes of the p_H range being investigated. Thus, when it was desired to study the p_H-activity curve of the enzyme in presence of $M/5$ phosphate between p_H 4·5 and 9·5, two solutions were made up, each $M/4$ in phosphate and $M/8$ in fumarate, but one acid to p_H 4·5 and the other alkaline to p_H 9·5. The solutions were mixed in various proportions to give the required intermediate p_H values. 20 cc. of each mixture was taken, and to it were added 1 cc. of propyl alcohol, 2 cc. of bacterial suspension, and 2 cc. of distilled water, making the final volume 25 cc. and the concentrations of phosphate and fumarate $M/5$ and $M/10$ respectively. The p_H of the final reaction mixture was then accurately measured initially and at the time of taking each sample. When the p_H was below 8, it was measured electrometrically by the quinhydrone method. Unfortunately the hydrogen electrode is useless in solutions of fumaric acid, so that the more alkaline p_H values had to be estimated by means of indicators. Since many of the reaction mixtures were necessarily unbuffered, the p_H often changed appreciably during the course of the incubation, and the average of the final and initial readings was assumed to be the p_H during the experiment. But when the difference between the initial and final p_H exceeded 0·1, the experiment was rejected, except in the alkaline citrate mixtures, as noted below.

RESULTS.

The effect of p_H on the activity of the enzyme in the absence of added salts is shown by the full line in Fig. 1. The various p_H values were obtained by neutralising the $M/10$ fumaric acid to different extents with NaOH. It is of course impossible to determine the activity of the enzyme in a completely salt-free medium, since the substrate is itself a salt. It will be seen that a symmetrical curve is obtained, covering the range between p_H 5 and 7·7, with an optimum at about 6·4.

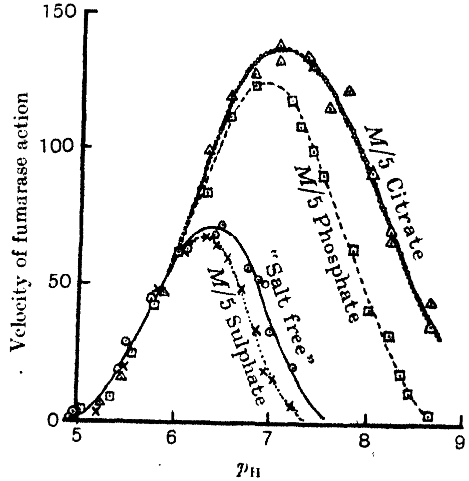

Fig. 1.

Between p_H 6 and 8·8, the addition of phosphates increases the reaction velocity. At any given p_H in this range, as the phosphate concentration is increased the activation is also increased, until a maximum effect is obtained which is the same for all higher phosphate concentrations. The results of a series at p_H 7·4 are shown in Fig. 2. It will be seen that the curve is approximately a rectangular hyperbola, suggesting that the salt combines with the enzyme, to give a more active enzyme-phosphate complex. The apparent Michaelis constant of the phosphate (*i.e.* the concentration at which half the maximum effect is obtained) at this p_H is about 0·0076 M, and the affinity (the reciprocal of the Michaelis constant) about 132. In order to relate the various curves to one another, the maximum velocity in presence of phosphate at p_H 7·4 for each enzyme preparation was taken as 100. In each series of reaction mixtures, a control was included containing the same amount of bacterial suspension in presence of $M/10$ fumaric acid and $M/5$ phosphate at

p_H 7·4, and the velocities in all the other reaction mixtures were expressed as percentages of the velocity in this control. In this way the results of all the series were made quantitatively comparable.

That an ion of the phosphate combines with the enzyme is made to appear more likely by the results at other p_H values. At 6·6 the phosphate-free enzyme itself has a considerable activity, the velocity being slightly under 50. The maximum activity induced by phosphate is about 100. The effect of varying phosphate concentration at this p_H is shown in Fig. 3. The curve is of the same type as that obtained at p_H 7·4, but the Michaelis constant is now 0·026 M and the affinity only about 38. Similar curves were obtained at p_H 7·0 and 8·0. The maximum velocity at p_H 7 is about 125 and at 8 about 45. The four curves are shown in Fig. 4 reduced to the same scale, the relative increase in velocity being plotted against phosphate concentration. The figure brings out clearly the regular rise of the affinity with p_H. In Fig. 5 is shown

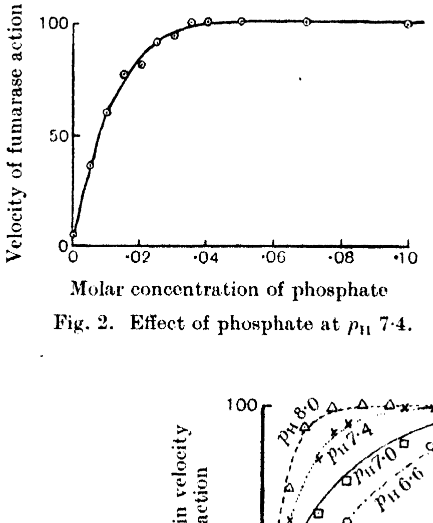

Fig. 2. Effect of phosphate at p_H 7·4.

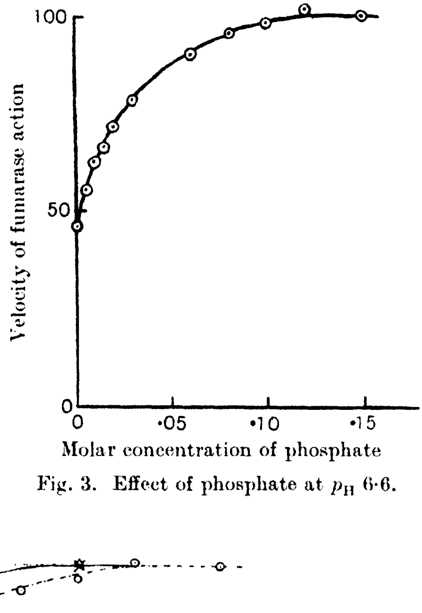

Fig. 3. Effect of phosphate at p_H 6·6.

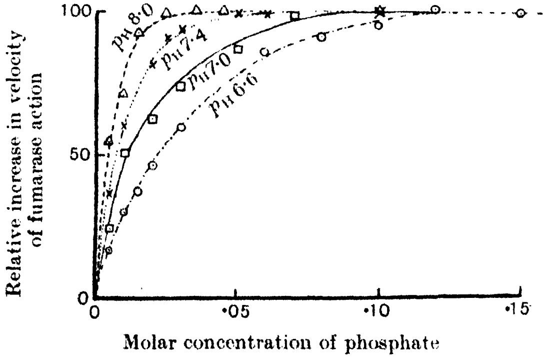

Fig. 4.

the affinity plotted against p_H. The curve shown is the dissociation curve for the reaction $H_2PO_4' \rightleftharpoons HPO_4'' + H^{\cdot}$, on to which the affinity at p_H 7·0 has been fitted. It will be seen that the other three points fall very well on the curve. The affinity at any p_H thus seems to be proportional to the concentration of HPO_4'' ions in the solution, making it seem extremely probable that the activating effect is due to an actual combination of the enzyme with this ion.

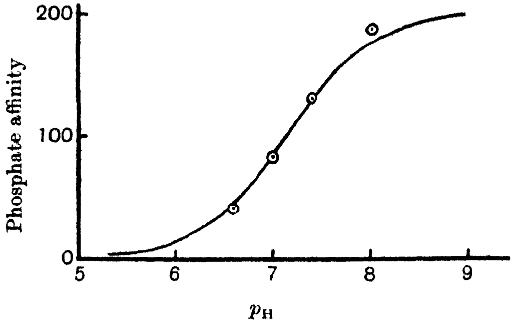

Fig. 5.

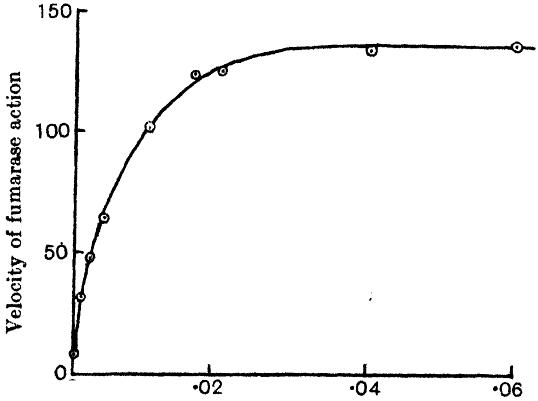

Fig. 6. Effect of citrate at p_H 7·4.

When the phosphate concentration is sufficient to give the maximum activation at any p_H, the enzyme may be regarded as saturated or completely combined with the phosphate ion. A phosphate concentration of $M/5$ is sufficient to effect this over the whole of the significant p_H range. A p_H-activity curve was therefore obtained in presence of $M/5$ phosphate, and is shown in Fig. 1. It will be seen that it is a symmetrical curve, which coincides with the "salt-free" curve between p_H 5 and 6, but then rises above it to an optimum at about p_H 6·9, after which it falls, roughly parallel to the alkaline limb of the "salt-free" curve to reach the horizontal axis at about p_H 8·8.

Citrate behaves similarly to phosphate, but gives a greater activation. Fig. 6 shows the effect of varying concentrations of citrate on the reaction velocity at p_H 7·4. It will be seen that the maximum velocity at this p_H is about 135, as against 100 for phosphate. The effect of p_H on the citrate affinity is the opposite of that on the phosphate, the affinity rising as the p_H is decreased. The results so far obtained suggest that it is the doubly ionised citrate which is effective, but this is not yet fully worked out. *M*/5 citrate gives maximum activation at all p_H values, and a p_H-activity curve in presence of this concentration is shown in Fig. 1. The curve is similar to that obtained with phosphate, but lies a little outside it over the alkaline range, the fumarase-citrate optimum being at about p_H 7·1. Since neither fumarates nor citrates are buffers over the alkaline range of this curve, the p_H of the reaction mixtures often changed greatly. Consequently a wider latitude than 0·1 p_H had to be allowed for this part of the curve, and the individual points are therefore rather scattered, although the curve as given is probably not far from correct.

Sulphates have a retarding effect over the alkaline part of the "salt-free" curve. *M*/5 sulphate gives the maximum effect, and the fumarase-sulphate p_H-activity curve is also shown in Fig. 1, the optimum being at about p_H 6·2.

The four p_H curves shown in Fig. 1 are related to one another like the dissociation residue curves of an ampholyte, whose acid p_K remains constant but whose alkaline p_K is shifted to different extents by the combination of the various anions. The results are completely explained by the hypothesis that the enzyme is an ampholyte which is active only in its isoelectric form. The enzyme can combine with various bivalent anions, the enzyme-anion complex having an altered dissociation constant for the $H^{\cdot}$ ion. Phosphates and citrates repress the ionisation, thus increasing the proportion of the enzyme molecules in the active form at any p_H in a given range, without affecting the rate at which an active enzyme molecule catalyses the hydration of the substrate. Sulphates encourage the ionisation, thus decreasing the number of active enzyme units, and so retarding the reaction velocity. Thus the effects of salts on fumarase are precisely similar to those found by Myrbäck for salivary amylase.

Preliminary experiments have been made with several other salts, and also on the combined effects of two or more salts. The power of affecting the velocity of fumarase action seems to be possessed by very many salts, and it is proposed to report the results of further studies in another communication.

SUMMARY.

1. "Resting" *B. coli communis*, washed free of all salts and suspended in distilled water, in presence of 2 or 4 % propyl alcohol will convert fumaric acid into its equilibrium mixture with *l*-malic acid at a linear rate for the greater part of the reaction. In the absence of added salts, the range of activity is between p_H 5 and 7·7, the p_H-activity curve being symmetrical, with an optimum at about 6·4.

2. Phosphates accelerate between p_H 6 and 8·8. If at any given p_H the increase in velocity is plotted against phosphate concentration, a rectangular hyperbola ("Michaelis curve") is obtained, suggesting that the phosphate combines with the enzyme non-competitively with the substrate. The "affinity" of the enzyme for phosphate at any given p_H is proportional to the HPO_4'' ion concentration. In presence of 0·2 *M* phosphate, which gives the maximum effect at every p_H, a symmetrical p_H-activity curve is obtained, with an optimum at 6·9.

3. Citrates behave similarly, giving a greater activation at every p_H, and an optimum at 7·1.

4. Sulphates depress the activity, giving an optimum at p_H 6·2.

5. The four p_H curves are related to one another like the dissociation residue curves of an ampholyte, in which the p_K of the OH' ion is constant, but the p_K of the $H^{\cdot}$ ion is shifted to different extents by the added anions. It seems, therefore, that the enzyme is only active when in the isoelectric state, the salt effect being due to an alteration in the proportion of the enzyme molecules in this active state at any given p_H.

It is a pleasure to express our thanks to Sir F. G. Hopkins and Mr J. B. S. Haldane for their interest and encouragement during the course of this work.

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LII. THE CHEMISTRY OF OESTRIN.

III. AN IMPROVED METHOD OF PREPARATION AND THE ISOLATION OF ACTIVE CRYSTALLINE MATERIAL.

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INTRODUCTION.

THE preparation of oestrus-producing concentrates from any source may be said to consist essentially of two main processes: (*a*) the extraction of the hormone together with fatty substances from the raw material and the removal of the bulk of the saponifiable fat from the extract, and (*b*) the purification of the resulting concentrate.

In previous communications [Marrian, 1929, 1, 2] such methods have been described. The part of the process which may be said to fall under (*a*) was carried out by purely empirical methods and it was emphasised that the yields could not be considered to approach the maximum possible. The subsequent processes were studied in a quantitative manner and it was shown that the extracts could be considerably purified in several ways with no detectable loss of potency.

In this work the initial stages of the process have been modified in view of the now generally accepted belief that oestrin is acidic in character [Funk, 1929; Marrian, 1929, 2; Butenandt, 1929, 1, 2; Laqueur, Dingemanse and Kober, 1930], and of the author's finding that the hormone can be apparently displaced from its alkali salts by carbon dioxide [Marrian, 1929, 2]. The aim throughout has been to obtain greater yields of active material from the urine of pregnancy, and to concentrate the extracts with the minimum loss to a stage from which the final isolation of the hormone could be attempted.

For this purpose a series of small batches of urine have been extracted and purified separately; accurate assays of the potency being made on the initial extract and the product in each case. These concentrates were combined and an attempt was made to effect a further purification. This was successful in that highly active crystalline material appearing to be a single substance was isolated.

Since this work was commenced Butenandt [1929, 1, 2] and Laqueur, Dingemanse and Kober [1930] have reported the isolation of active crystalline materials from urine which, judging from the combustion results, appear to be identical. The latter workers, however, are doubtful if this substance is actually the pure hormone itself. In both cases the actual methods of isolation are not given in detail, and no mention is made of the yields obtained. According to these authors Doisy also described the preparation of an active crystalline substance at the Physiological Congress in Boston last summer. So far, however, no details of Doisy's work have reached the author. Wieland, Straub and Dorfmuller [1929] have also reported the isolation of an active crystalline substance which, however, does not appear to approach purity.

EXPERIMENTAL.

Method of assay. A standard technique for assaying the activity of the various fractions was employed throughout the work. The fraction to be tested was dried, weighed and made up to a volume of 500 cc. in a mixture of equal parts of alcohol and chloroform. 0·5 cc. of this solution was withdrawn and diluted to 10 cc. with alcohol. A small volume of such a testing sample, usually 0·1 cc. to 1·0 cc. depending upon the amount of active material expected to be present, was withdrawn and evaporated to dryness in a 10 cc. volumetric flask. The residue was dissolved in 0·5 cc. alcohol and made up to 10 cc. with water. This solution was then injected into seven ovariectomised mice in doses graded from 4 × 0·05 cc. to 4 × 0·5 cc., each dose being given in four injections at 12-hour intervals. From such preliminary tests an approximate idea could be formed of the activity of the fraction.

With this information a fresh aqueous dilution of an aliquot portion of the testing sample was made and injected into a group of twenty mice in four doses of 0·1 cc., the activity being determined in the manner described in previous papers [Marrian and Parkes, 1929; Marrian, 1929, 1, 2].

Provided that not more than 70 % and not less than 15 % of the mice showed an oestrous response, the results were satisfactorily accurate. When the response did not fall within these limits, as was sometimes the case with such rough preliminary tests, it was thought desirable to repeat the tests.

The use of a small amount of alcohol to obtain an "aqueous solution" has not been found to affect the accuracy of the results and no ill effects on the test-animals have been observed in consequence. In any case, this method seems preferable to that adopted by Butenandt [1929, 2], in which the active material is injected in a solution of sesame oil. The repeated injection of oily solutions into mice almost invariably leads to severe subcutaneous granulations, which may result in the incomplete absorption of subsequent injections.

Collection and extraction of urine. The urine was collected each day in vessels containing a small quantity of toluene, acidified with concentrated HCl in the proportion of 5 cc. to 1 litre, and extracted four times with ether.

The ethereal extracts were washed once with water, evaporated to dryness and stored in alcoholic solution at 0°.

Saponification of the crude extract. When 50 litres of urine had been extracted, the alcoholic solution of the crude ether-soluble material was evaporated to dryness and heated with 400 cc. of 5 % aqueous potassium hydroxide solution in a boiling water-bath for 30 minutes. Carbon dioxide was then passed into the mixture for 3 hours. After dilution with an equal volume of water, the mixture was extracted twelve times with successive portions of 100 cc. ether. The combined ethereal extracts were washed four times with 50 cc. lots of 0·2 *N* HCl, twice with the same amounts of water and then evaporated to dryness.

Extraction with cold acetone. In a previous paper [Marrian, 1929, 1] it was shown that the unsaponifiable matter of the urine of pregnancy contained small amounts of a substance melting at about 233–234° which was believed to be a dihydroxy-alcohol.

Although the removal of this substance is probably unnecessary at this stage of the purification process, more of the substance was required for further examination. It was shown that this alcohol could be removed from the unsaponifiable matter by extraction with ice-cold acetone or ether, in either of which it is fairly insoluble. Since oestrin, according to recent work, has a low solubility in ether, the use of acetone seemed preferable for the purpose.

The dry unsaponifiable matter was dissolved in 30 cc. of boiling acetone and left at 0° for 12 hours. The flask was then immersed in an ice-salt freezing mixture for 1 hour to complete the separation of the alcohol, and the mixture filtered through an ordinary filter. The flask and filter were washed with a further 10 cc. of cold acetone. The residue in the filter was then washed back into the original flask with a boiling mixture of chloroform and alcohol. This was evaporated to dryness and treated with 15 cc. of acetone in precisely the same manner to remove remaining traces of the hormone. The combined acetone extracts, on evaporation to dryness, yielded a reddish brown gum.

Extraction with 50 % alcohol. The acetone-soluble fraction was heated for 5–10 minutes to boiling with 30 cc. of 50 % alcohol, and then kept at 0°. The next day the mixture was filtered, the flask and filter being washed with another 10 cc. of ice-cold 50 % alcohol. The insoluble material on the filter was washed back into the original flask with a hot alcohol-chloroform mixture. This solution, after evaporation to dryness, was treated with 20 cc. of 50 % alcohol with another washing of 10 cc. in the same way. The latter process was repeated twice, making in all four extractions with 50 % alcohol.

The combined 50 % alcohol filtrates were evaporated to dryness.

Extraction from ethereal solution with aqueous alkali. It was shown in a previous paper [Marrian, 1929, 2] that, owing to the acidic properties of oestrin, a considerable degree of purification could be attained by extraction of an ethereal solution of the hormone with aqueous alkali. By acidification and ether extraction the oestrin could be recovered quantitatively from the alkaline solution.

The fraction soluble in 50 % alcohol was dissolved in about 10 cc. of alcohol and then diluted with 700 cc. of ether. The low solubility of this material in ether did not permit direct solution. This solution was then extracted ten times with successive 50 cc. lots of 5 % aqueous potassium hydroxide. The combined alkali extracts were acidified with 110 cc. of 12 *N* HCl and extracted eight times with 100 cc. portions of ether. The combined ethereal extracts were washed three times with water and evaporated to dryness.

The final product varied considerably in colour and appearance from batch to batch, but in general it may be described as a brown gum containing varying amounts of what appeared to be crystalline material.

The results of the whole method of concentration are shown in Table I. These results clearly indicate that by these methods very large yields of active material can be obtained and that the initial extracts can be enormously reduced in bulk with only about 40 % loss of activity.

Each fraction of batch P.U. 7A was assayed accurately to determine at which stage of the purification this loss occurred. The results given in Table II definitely show that practically the whole of the loss occurs during the saponification. The extraordinary uniformity of the extent of this loss, with the exception of batch P.U. 7C, suggests that incomplete extraction by the ether is not the cause. This point is now being investigated.

Table I.

	Batch No. ...	P.U. 7A	P.U. 7B	P.U. 7C	P.U. 7D	P.U. 7E	Average
	Vol. urine (litres)	50	50	50	50	50	50
Initial acid ether extract	Wt. (g.)	22	21	21	18	20	20
	No. m.u.	2,069,000	980,000	850,000	444,000	792,000	1,027,000
	Yield, m.u. per litre	41,400	19,600	17,000	8,880	15,800	20,500
Final product	Wt. (g.)	0·364	0·253	0·471	1·107	0·516	0·342
	No. m.u.	1,208,000	546,000	614,000	267,000	445,000	616,000
	Yield, m.u. per litre	24,160	10,920	12,280	5,340	8,900	12,300
	Wt. 1 m.u. (mg.)	0·000301	0·000463	0·000767	0·000399	0·00116	0·000618
Loss in purification (%)		42	44	28	38	44	40

Table II. *Batch P.U. 7A.*

	Wt. (g.)	No. m.u.	Loss in m.u. (%)
Initial ether extract	22	2,069,000	—
"CO_2" unsaponifiable matter ...	2·845	1,278,000	38
Acetone-soluble	2·456	1,236,000	40
50 % alcohol-soluble	0·742	1,236,000	40
Alkali extract from ether solution	0·364	1,208,000	42

Extraction by ether from alkaline solution. According to Funk [1929] the alkali metal salts of oestrin possess a definite solubility in ether and on this account the hormone is present in the unsaponifiable matter from active fatty material. If this is the case, it should be possible, by thorough extraction with ether from alkaline solution, to remove all the activity.

Since the use of carbon dioxide in the saponification of these batches results in the presence of a large amount of material that would not be present in the unsaponifiable matter if prepared by ordinary methods, it was thought that repeated extraction of an alkaline solution by ether, without the use of carbon dioxide, might prove to be an efficient method of purification.

The whole of batch B was used to try out this method. The material, weighing 0·253 g. and containing 546,000 mouse units, was treated with 400 cc. of 5 % aqueous potassium hydroxide in which it readily dissolved. This solution was diluted with 300 cc. of water and then extracted twenty times with 100 cc. lots of ether. The combined ethereal extracts were washed first with 0·5 *N* HCl and then three times with water, and on evaporation yielded 0·0588 g. of solid material. On assaying the activity of this fraction, it was found that only 82,400 mouse units had been extracted. This corresponds to an activity of 1 m.u. = 0·000704 mg. as against the activity of the original material of 1 m.u. = 0·000403 mg. This method therefore is entirely worthless for the further purification of these extracts, and it seems extremely doubtful if the alkali salt of the hormone is appreciably soluble in ether. It seems more probable that the presence of oestrin in unsaponifiable matter is due to adsorption on the ether-soluble material.

The active material was recovered from the extracted alkaline solution by acidification and ether extraction, the two fractions being combined and re-tested.

Preparation of water-soluble extract. With part of the material obtained by the recombination of the fractions from the last experiment, an attempt was made to prepare an aqueous solution of such potency as might be useful for clinical work.

The material containing 235,000 mouse units was treated with 25 cc. of hot 0·1 *N* NaOH. The main bulk dissolved, yielding a deep red-brown solution containing a small amount of finely divided material in suspension. This solution was then neutralised carefully with 0·1 *N* HCl, using phenolphthalein as external indicator. By careful adjustment a point was reached where the solution was just acid to phenolphthalein, but no precipitation occurred. 0·25 cc. of "tricresol" was added and after making up to a volume of 250 cc. with water, the solution was left standing in a tall cylinder to allow the few suspended particles to flocculate and settle.

On assaying a portion of the clear supernatant liquid, which had a light brown colour, it was found to contain 670 mouse units per cc., so that the total activity corresponded to 168,000 mouse units. The remaining 67,000 mouse units were presumably adsorbed on the small amount of insoluble material.

Isolation of active crystalline material.

Butenandt [1929, 2] observed that the active crystalline substance isolated by him had a low solubility in ether. This fact suggested the possibility of using ether or a mixture of ether and some other solvent for the separation of solid material from the urine concentrates.

In preliminary experiments direct crystallisation from ether was found to be impracticable, owing to the low solubility of the concentrates even in large volumes of boiling ether. By solution in a very small volume of alcohol and addition of an excess of ether, however, a large amount of solid material could be precipitated.

The combined concentrates from batches P.U. A, C, D and E, weighing 1·457 g. and containing 2,534,000 mouse units, were treated in this way. The dried material was warmed for a short time with 0·75 cc. of alcohol, when it softened to a mobile oily consistency. To this were added 10 cc. of ether, when a heavy granular precipitate was thrown down. The flask was placed in an ice-salt freezing mixture for 45 minutes, and then filtered rapidly on a small Büchner funnel. The filtrate was evaporated to dryness and again treated with alcohol and ether in the same manner, when a further small amount of solid was separated. The combined solid precipitates were washed with a few cc. of alcohol-ether mixture to remove as much of the more soluble gummy material as possible.

This solid material was light brown in colour and weighed 0·1442 g. On assaying, it was found to contain 1,233,000 mouse units, or about half of the total, corresponding to an activity of 1 m.u. = 0·000116 mg. This is slightly more potent than the concentrate described by the author in a previous paper [Marrian, 1929, 2].

The material was still too highly pigmented for purification by means of recrystallisation to be effected. An attempt was therefore made to clean it by treatment with charcoal. The solid matter was dissolved in 20 cc. of alcohol and boiled under a reflux condenser with a small amount of "norite" for 30 minutes. The mixture was filtered hot, the flask and filter being washed with four successive 10 cc. portions of boiling alcohol. The alcoholic filtrate was evaporated to dryness and the process repeated a second time.

The product was now nearly white in colour. The weight was 0·1439 g., showing that the pigment removed formed only a small fraction of the total bulk. It contained 1,050,000 mouse units, corresponding to an activity of 1 m.u. = 0·000137 mg. It is doubtful if this apparent slight decrease in potency is significant. The difference of about 13 % is only slightly outside what is believed to be the probable error in the method of assay. Preliminary experiments showed that this material, which was amorphous, could be crystallised from aqueous methyl alcohol. It was therefore dissolved in 10 cc. of boiling 57 % methyl alcohol and left overnight at 0°. 0·0767 g. of crystalline material were obtained in this way. The crystals were still slightly brown in colour, but the bulk of the pigment appeared to be in the mother liquor. The recrystallisation was repeated with 5 cc. 57 % methyl alcohol. The twice recrystallised material weighed 0·0613 g. Two tests on this gave totals of 498,000 and 556,000 mouse units, corresponding to an average activity of 1 m.u. = 0·000116 mg. The potency is not considered to be significantly different from that of the original non-crystalline solid.

The mother liquors from the two crystallisations were evaporated to dryness and treated with 12 cc. of boiling 42 % methyl alcohol. On cooling to 0° a further crop of crystals was obtained, which were rather more pigmented than those of the first batch. This material weighed 0·0275 g. and contained 256,000 m.u., corresponding to an activity of 1 m.u. = 0·000108 mg.

The mother liquor from this recrystallisation contained less than 125,000 mouse units.

Properties of the crystalline substance.

The first batch of crystals were very slightly pigmented. On microscopic examination two types of crystals were observed (Plate II A). It was shown, however, that these two types were merely different forms of the same substance. On recrystallisation from 57 % methyl alcohol at 0°, the crystals were all of the type shown in Plate II B. On recrystallisation at ordinary temperatures, the crystalline form was of the second type shown in Plate II A.

The substance appeared to have no definite melting point. From 222° onwards a slight darkening and shrinkage were observed. At 256–260° the substance melted completely and simultaneously decomposed.

On treating a small quantity of the substance in chloroform solution with H_2SO_4 and acetic anhydride (Liebermann-Burchardt reaction) a pale orange-yellow colour with a marked green fluorescence resulted. This is very similar to the colour reaction observed by Wieland, Straub and Dorfmuller [1929] with their preparations. Butenandt [1929, 1], on the other hand, observed no characteristic colour reaction with these reagents.

A preliminary micro-combustion and molecular weight determination showed that the substance contained three oxygen atoms to about eighteen carbon atoms. The percentage of carbon was somewhat lower than that in the substances isolated by Butenandt [1929, 1, 2] and Laqueur, Dingemanse and Kober [1930]. In view of the fact that there is at present no satisfactory criterion of purity, these results are probably only of value in so far as they indicate the approximate molecular dimensions. The close agreement between the observed value for the molecular weight and the figure corresponding to the formula suggested by the analysis, is probably fortuitous.

Nitrogen could not be detected in the substance.

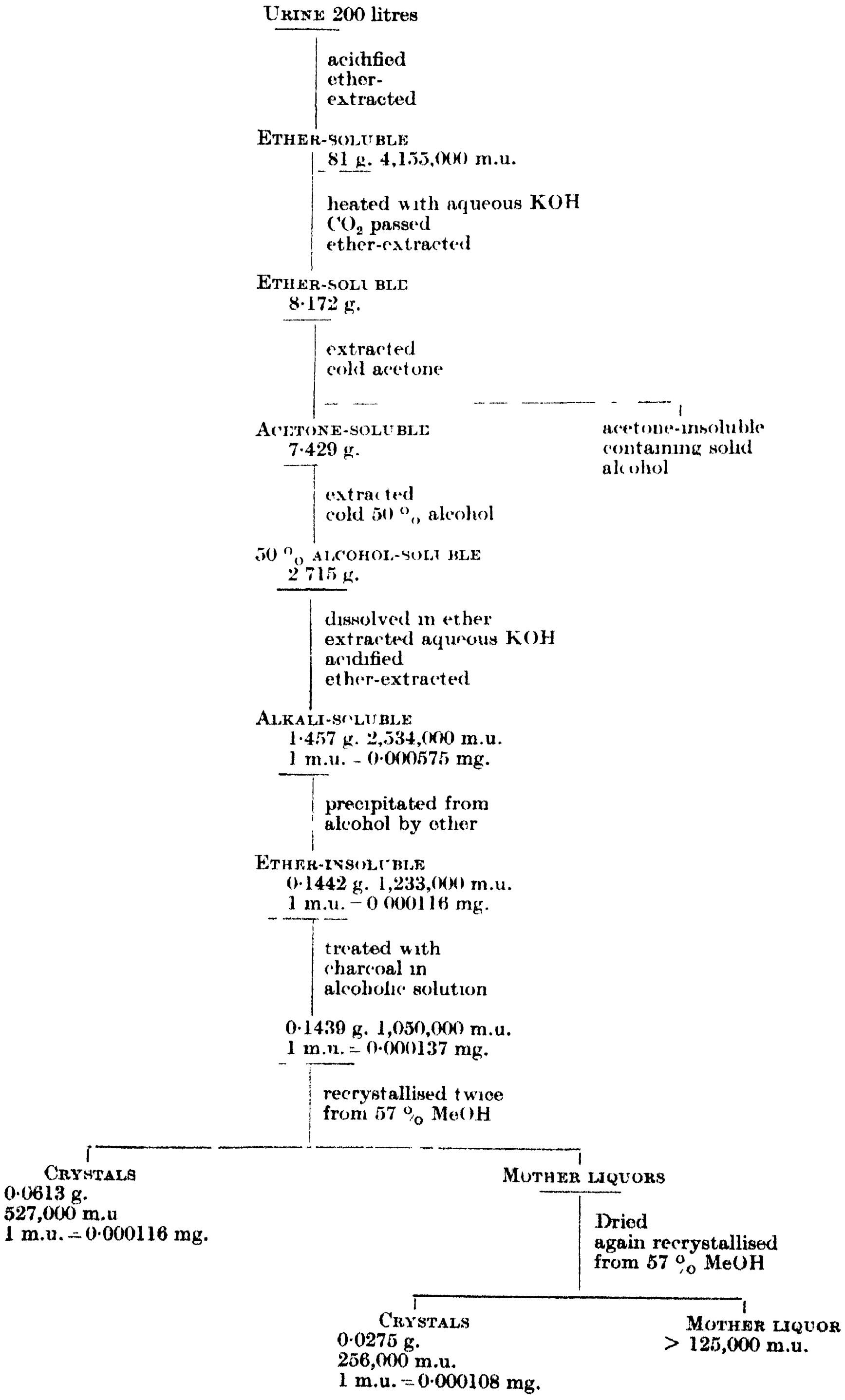
4·541 mg. gave 12·415 mg. CO_2 and 3·50 mg. H_2O.

C 74·53 %, H 8·56 %.

Mol. wt. by Rast's camphor method: 288, 289.

Calculated for $C_{18}H_{24}O_3$: C 75·00 %, H 8·33 %, molecular weight 288.

Table III. *The purification of batches P.U. 7* A, C, D *and* E.



URINE 200 litres

acidified ether-extracted

ETHER-SOLUBLE
81 g. 4,155,000 m.u.

heated with aqueous KOH
CO_2 passed
ether-extracted

ETHER-SOLUBLE
8·172 g.

extracted cold acetone

ACETONE-SOLUBLE
7·429 g.

acetone-insoluble containing solid alcohol

extracted cold 50 % alcohol

50 % ALCOHOL-SOLUBLE
2·715 g.

dissolved in ether
extracted aqueous KOH
acidified
ether-extracted

ALKALI-SOLUBLE
1·457 g. 2,534,000 m.u.
1 m.u. = 0·000575 mg.

precipitated from alcohol by ether

ETHER-INSOLUBLE
0·1442 g. 1,233,000 m.u.
1 m.u. = 0·000116 mg.

treated with charcoal in alcoholic solution

0·1439 g. 1,050,000 m.u.
1 m.u. = 0·000137 mg.

recrystallised twice from 57 % MeOH

CRYSTALS
0·0613 g.
527,000 m.u
1 m.u. = 0·000116 mg.

MOTHER LIQUORS

Dried
again recrystallised
from 57 % MeOH

CRYSTALS
0·0275 g.
256,000 m.u.
1 m.u. = 0·000108 mg.

MOTHER LIQUOR
> 125,000 m.u.

DISCUSSION.

By the methods described in the earlier part of the paper, very large yields of oestrin can be obtained in a concentrated form from the urine of pregnancy with a relatively small loss of total activity. Such concentrates make a suitable starting point for the preparation of aqueous solutions suitable for clinical or physiological use. The advantage of the method of obtaining aqueous solutions of the hormone, as described in this paper, over methods previously in general use, is that far higher concentrations of the active substance can easily be obtained. The only limit appears to be the solubility of the alkali salts of the hormone and of the substances associated with the hormone. In the older methods, the very much lower solubility of the free acidic substances in water was the limiting factor. The importance of such highly concentrated aqueous solutions for physiological and clinical use is made clear by recent work carried out in collaboration with Dr A. S. Parkes [Marrian and Parkes, 1930].

Two points in connection with the crystalline substance are at present not clear. In the first place, are the active crystalline compounds isolated by Butenandt [1929, 1, 2], Laqueur, Dingemanse and Kober [1930] and the author identical? In the second place, are any of these substances the pure hormone itself, or are they merely inactive substances on which is adsorbed a small amount of the active hormone?

Butenandt [1929, 2] observed that his active substance melted with decomposition at 240°, Laqueur, Dingemanse and Kober [1930] do not record a melting point for their substance[1], while the author's preparation melted very indefinitely with decomposition at 256–261°. The decomposition which occurs makes it obvious that the melting points cannot be accepted as satisfactory criteria of purity.

The close agreement between the combustion figures of the first two preparations suggests that these two substances may be identical (Butenandt, C 78·31 %; H 8·13 %; Laqueur, Dingemanse and Kober, C 78·61 %; H 8·25 %). A somewhat lower figure for the carbon content was, however, obtained for the author's preparation.

Butenandt reports an activity of 8 million mouse units per g., Laqueur, Dingemanse and Kober 8–10 million per g., while the author's preparation has also an activity of 8 million mouse units per g. These figures, however, are not so uniform as they might at first appear, as the methods of testing are different in each case. Laqueur and de Jongh [1929] test their materials in six doses spread over a period of 48 hours. A similar technique has been adopted by Allan, Dickens and Dodds [1930]. The latter show that this method gives approximately a 25 % higher response than that of giving four doses over 36 hours. Experiments confirming this relationship between the two methods have been carried out by the author.

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[1] Shortly after going to press, a more detailed paper appeared in *Deutsch. med. Woch.* from Laqueur's laboratory, in which the melting point of crystalline "Menformon" is given as 240°: decomposition is not mentioned.

Butenandt [1929, 2], on the other hand, administered his preparations as a single dose in sesame oil solution. In later work [Butenandt and Ziegner, 1930], he shows that when four and six injections are administered over 36 and 40 hours respectively, the apparent activity of the preparation is raised from 8 to 30 and 40 million mouse units per g. respectively. These results, while providing further data on the relationship between the four and six dose methods, are of little value for deciding the point at issue, namely, the relationship between the activity of a preparation given in a single dose in oil solution and that of a multiple dose in what is virtually an aqueous solution. Allan, Dickens and Dodds [1930] show the relationship between single and multiple doses in aqueous solution and also single doses in an oil-water emulsion. The latter is, however, hardly the same as a true oil solution. Rapid absorption of the hormone must occur directly from the aqueous phase.

A few experiments have been carried out by the author bearing on this question. Graded doses of the active crystalline material dissolved in sesame oil were administered to a few mice by the method in which four injections are given. Too much reliance cannot be placed on the results, since groups of twenty were not used on account of the adverse effects of the oil injections. The results indicated, however, that the material had at least twice the apparent activity displayed when injected in aqueous solution. Thus, tested by this method, the crystalline material has an activity of about 16 million units per g. This, although lower than Butenandt's figure of 30 millions, is more nearly of the same order.

The remaining discrepancy might be accounted for by a difference in the total volume of oil injected. Marrian and Parkes [1929] showed that, with aqueous solutions, the volume of the dose had no appreciable effect upon the apparent activity. With oil solutions this cannot be the case. Within certain limits the activity of a preparation varies inversely with its rate of absorption when administered subcutaneously. Absorption from a large volume of oil must necessarily be slower than from a small volume, and an increased apparent activity may result with the larger volume. With still larger volumes of oil, the rate of absorption must be still further slowed and then presumably a decrease in apparent activity would be observed. With aqueous solutions the rate of absorption is so rapid that these considerations do not apply.

It is considered, therefore, that the apparent difference between the activity of Butenandt's material and the one described here is not so great as might at first appear.

Whether these crystalline substances are actually the hormone itself is a point that can only be decided by future work. Laqueur, Dingemanse and Kober [1930] are doubtful on this point, since some of their preparations showed an activity of as much as 14 million mouse units per g. Until the activity has been shown to remain constant after repeated recrystallisations from different solvents, no conclusions should be drawn.

B

Active crystalline substance isolated from the urine of pregnancy

SUMMARY.

1. By acidification and ether-extraction of urine from pregnant women, yields of oestrin varying from 8000 to 40,000 mouse units per litre can be obtained.

2. Methods are described by which these extracts can be greatly purified with a loss of only 40 % of the activity throughout the process. This loss occurs entirely in the initial stage of saponification. The mouse unit of these purified preparations is of the order of 0·0006 mg.

3. From such purified preparations an active crystalline substance has been isolated. The mouse unit of this substance is about 0·00011 mg.

4. The possible identity of this substance with the active substances recently isolated by Butenandt and by Laqueur, Dingemanse and Kober is discussed.

5. There is no proof at present that this substance is actually the pure hormone.

The author wishes to express his gratitude to Prof. J. C. Drummond for his continued interest and advice, to Dr A. S. Parkes for the large number of ovariectomised mice, without which this work would have been impossible, and to Prof. F. J. Browne for providing facilities for the collection of urine from University College Hospital.

The expenses of this work were defrayed by a grant from the Medical Research Council, to whom the author wishes to express his thanks.

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LIII. STUDIES ON ANTIGLYOXALASE.

I. THE ACTION OF PANCREATIC EXTRACT ON PHENYLGLYOXAL.

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(*Received March 3rd, 1930.*)

While investigating the distribution of glyoxalase in various animal tissues, Dakin and Dudley [1913] found that the pancreas is distinguished from all other animal organs in that it not only contains no glyoxalase itself, but also inhibits the glyoxalase action of other tissues with which it is incubated. They assumed the existence in pancreas of an inhibiting substance which they called antiglyoxalase. Some further work on antiglyoxalase has been published by the same authors [1914], by Foster [1925], by Kuhn and Heckscher [1926] and by Ariyama [1928]. Nothing definite is known, however, about the nature and the mode of action of antiglyoxalase, and the interest which attaches to the transformations of methylglyoxal as probable important steps in the intermediary metabolism of carbohydrates seemed to warrant a further investigation of the problem. Both Dakin and Dudley, and Ariyama are agreed that the inhibiting action of antiglyoxalase develops gradually when the pancreatic extract is incubated together with a glyoxalase solution. The fullest inhibition is only obtained when the phenyl- or methylglyoxal is added after several hours' incubation of glyoxalase with antiglyoxalase. This seems to indicate that the inhibition is due to a reaction of the inhibiting factor with the enzyme, but it is not possible to decide on the present evidence whether glyoxalase is destroyed in the process or merely inactivated by combination with antiglyoxalase. Foster came to a different conclusion regarding the mode of action of antiglyoxalase. This author observed that whenever phenylglyoxal is incubated with pancreas, with or without addition of other tissue extracts, an orange colour develops in the solution. Owing to an apparent parallelism between the intensity of the colour developed and the intensity of the inhibition found, she suggested that the inhibiting action of pancreas might be due to the combination of some substance present in it with the glyoxal, forming a coloured compound resistant to the action of glyoxalase. The action of antiglyoxalase would thus be not on the enzyme but on the substrate. While the present work fully confirms Foster's observation, it will be seen that the phenomenon described by her is entirely independent of the antiglyoxalase action of pancreas.

TECHNIQUE.

Dakin and Dudley, and Foster followed the action of glyoxalase by estimating polarimetrically the mandelic acid formed. Kuhn and Heckscher estimated methylglyoxal in the solution by an iodimetric method and thus followed its disappearance, whilst Ariyama used for the same purpose a colorimetric method based on the reduction of Benedict's uric acid reagent by glyoxals in presence of KCN. It was thought that a manometric method, based on the principle of Warburg's method for following glycolysis, might prove more rapid and less laborious. Warburg's technique had already been used by Meyerhof [1925] for following the transformation of methylglyoxal into lactic acid by tissue slices. As no Warburg manometers were available and the work had to be done with manometers of the Barcroft type, it was first necessary to adapt the technique so as to allow it to be used with the Barcroft manometer. Warburg's method consists in measuring manometrically the CO_2 expelled from a solution containing $NaHCO_3$ by the lactic acid produced. To adjust the p_H it is necessary to equilibrate the solution against a CO_2 atmosphere, the CO_2 tension required depending on the $NaHCO_3$ concentration and on the p_H desired. The Barcroft manometer cannot be filled with a gas by passing it through the cups as in Warburg's method. Instead it is necessary to evacuate it and fill it with the gas required, a process which has to be repeated at least twice, to assure a fairly complete replacement of the air. Filling the manometers in this way with air-CO_2 mixtures did not lead to constant results, and the following technique was adopted after some trials.

The manometers are connected with the pump and with an aspirator bottle filled with CO_2 from a cylinder. They are also connected with a mercury manometer and with a gas burette, used for checking from time to time the purity of the CO_2. The Barcroft manometers are twice evacuated and filled with CO_2; thereupon they are again connected with the pump and the pressure in the whole system is lowered to the tension of CO_2 desired. The process is followed by means of the mercury manometer. A correction has of course to be applied for the difference in water-vapour tension in the Barcroft cups at 38° and in the connections with the mercury manometer at room temperature. Now the manometer cups are lowered into the thermostat in the usual way, and the shaking mechanism is started. After about 5 minutes the taps of the manometers are closed. Though pressures of about one-fifth of an atmosphere within the cups have been used most of the time, no trouble has been experienced with leakages. The $NaHCO_3$ and CO_2 concentrations required were calculated on the basis of the Henderson-Hasselbalch formula, modified by Hastings and Sendroy [1925] so as to take account of the activities. Four Barcroft manometers were used for each experiment, run as two duplicate pairs, a necessary precaution since duplicates, whilst usually agreeing within about 3–5 %, were occasionally found to diverge more widely. Both cups of the manometer contained the same amounts of water, $NaHCO_3$

solution, enzyme, etc. A small glass cup with a platinum hook was hung on the edge of the KOH tube of the manometer cup. That in the right-hand cup contained the glyoxal solution (usually 0·3–0·6 cc.) whilst that in the left cup contained an equal volume of water. After the taps have been closed, as described above, the initial reading is taken and the small cups are upset by slightly lifting the manometer and tapping with the finger.

RESULTS AND DISCUSSION.

Fig. 1 shows the effect of adding pancreatic extract to a glyoxalase solution in a Barcroft manometer. Minced liver, extracted with about five times its volume of water, was used (as also in the following experiments) as glyoxalase solution. The pancreatic extract was prepared in a similar manner. The curve without pancreas was obtained with 0·6 cc. liver extract in the cups, for the curve with pancreas 0·6 cc. liver extract and 1·0 cc. pancreatic extract were used. It will be seen that, instead of inhibiting, the pancreas seems at first actually to accelerate the reaction, and only after about 2 hours does the reaction velocity with pancreas begin to fall off compared to that with liver alone. Controls were thereupon made with pancreas alone, and it was found that this causes on addition of phenylglyoxal an evolution of CO_2, that is, an acid production, of the same order approximately as an equal volume of liver extract. Hence the effect described seems to be due to the action of the pancreas superposed on that of the liver-glyoxalase, rather than to an accelerating action of the former on the latter. Ariyama, measuring the methylglyoxal disappearance, also found an apparent acceleration at first after adding pancreas, and several hours were required before inhibition began to develop. He suggests that some glyoxalase may be present in pancreas, without bringing any evidence to support this suggestion, beyond the fact that some methylglyoxal disappears on incubation with pancreatin alone. To test whether the acid production on addition of phenylglyoxal to pancreatic extract is due to a transformation of the former into mandelic acid, pancreatic extract was incubated with phenylglyoxal in comparatively large scale

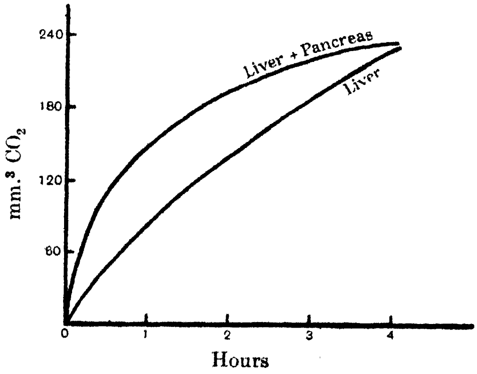

Fig. 1.

experiments and an attempt was made to isolate mandelic acid by the method described by Dakin and Dudley [1914]. Several such experiments were made, but all with completely negative results. Instead, it was found that, as described by Foster, an orange or brownish colour develops in the solution and on longer incubation with high initial phenylglyoxal concentrations a fairly copious yellow precipitate is deposited. The precipitate, after centrifuging off, was found to consist of a substance readily soluble in alcohol or ether but only very sparingly in water. Attempts to recrystallise it from ether proved fruitless, as a yellow oil remained which on standing gradually solidified to a resinous substance. That the action of the pancreatic extract on phenylglyoxal is not due to an enzyme follows from the fact that prolonged boiling or even autoclaving does not affect its activity. Even 2 hours' boiling with about 20 % HCl or KOH does not cause more than a 20 % decrease in activity. In Fig. 2 the effect of variations in the concentration of phenylglyoxal on the velocity of its reaction with pancreas is shown. The molar concentration of phenylglyoxal in the solution is plotted against the CO_2 (in $mm.^3$) evolved in 5 minutes. The shape of the curve suggests that two molecules of phenylglyoxal may enter into the reaction. On the other hand, the reaction velocity is strictly proportional to the amount of pancreatic extract taken. The effect of changing the p_H between 7·0 and 7·9 was investigated and a rapid rise in reaction velocity with increasing alkalinity was found. Below p_H 7·0 the reaction is negligible. The substance in pancreas, responsible for the reaction with phenylglyoxal, is destroyed by treating with $NaNO_2$ and acetic acid, which suggests that we are dealing with an amino-compound.

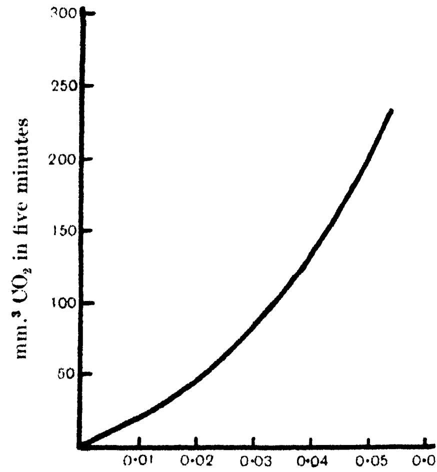

Fig. 2.

It is clear that any reaction involving the condensation of $—NH_2$ groups with a glyoxal must lead to an increase in the acidity of the solution and hence to an evolution of CO_2. The effect would somewhat resemble that made use of in Sørensen's method of amino-acid titration.

It is well known that phenylglyoxal reacts with ammonia and amines by condensation. Pinner [1902, 1905] identified the products of the action of ammonia on phenylglyoxal as consisting partly of 1-benzoyl-4-phenyliminazole and partly of 1 : 4-diphenyl-3-hydroxypyrazine. The products of the action of amino-acids on glyoxals have not yet been investigated. Neuberg and Kobel [1927] describe the action of amino-acids and related compounds on methylglyoxal. On heating the solution they observed extensive decomposition, with production of CO_2 and acetaldehyde. In the cold they only observed a change in rotation and formation of "melanin."

Attempts were made to isolate from the pancreas the substance or substances responsible for the reaction. It proved possible to precipitate with tannic acid much of the protein and peptone present in the extract, without markedly diminishing its power of reacting with phenylglyoxal. The tannic acid was removed by baryta. On treating the tannic acid filtrate with phosphotungstic acid the substances looked for were completely precipitated. This is evidence in favour of the supposition that we are dealing with amino-compounds. Repeated attempts to isolate these substances from the phosphotungstic acid precipitate by the usual procedure proved fruitless. The occurrence of the substances in question in other sources than pancreas was then investigated. It was found that liver, muscle and kidney are completely inactivated by 5 or 10 minutes' boiling. On the other hand, boiled yeast extract and boiled extract of testis were found to possess an activity similar to that of pancreas. Pancreas, yeast and testis are known to be particularly rich in histones, containing a high proportion of diamino-acids. On allowing phenylglyoxal to act on various amino-acids in Barcroft manometers, as described above, it was found that whilst monoamino-acids are inactive, the diamino-acids, histidine, arginine, and lysine give a reaction very similar to that obtained with pancreas. Both acid production and the development of an orange or yellow colour were observed.

Reacting as an aldehyde, phenylglyoxal should of course condense with monoamino-acids in a way analogous to the condensation obtained with formaldehyde. Using, in the Barcroft method described, formaldehyde or acetaldehyde, reaction rates too rapid to be measured were obtained both with mono- and with di-amino-acids. No colour develops in the solution when formaldehyde or acetaldehyde reacts with an amino-acid, and this, together with the fact that only diamino-acids react with phenylglyoxal seems to show that the two reactions are quite distinct. The curve shown in Fig. 2, indicating that two molecules of phenylglyoxal may enter the reaction, confirms this view. It is interesting to note that the substances obtained in this reaction somewhat resemble, in their physical properties, the products of the action of ammonia on phenylglyoxal, as described by Pinner. According to Mr Pirie (unpublished communication) chloral hydrate produces no shift of

p_H in a solution of alanine. It seems at any rate possible that the fact that phenylglyoxal exists as a hydrate is an important factor in determining the reactions described in this paper.

Whilst it has been shown that the diamino-acids give with phenylglyoxal a reaction similar to that obtained with pancreatic extract, the question remains whether the diamino-acids present in the extract are sufficient to account for the whole of the reaction obtained. A number of experiments were made, in which the amino-nitrogen and the reaction velocity with phenylglyoxal of a boiled pancreatic extract and of a histidine solution were compared. The histidine solution was found to possess only two-thirds of the activity of the pancreatic extract, whilst it contained 1·4 times as much amino-nitrogen. As the whole of the free amino-nitrogen of the pancreatic extract cannot be derived from diamino-acids the disparity of the reaction rate and the diamino-acid concentration must be still greater than is indicated by the above figures. No special search was made for other substances in pancreas giving the same reaction. But it was found that glycylglycine shows the reaction, and so does liver juice after partial acid hydrolysis. Hence it appears that polypeptides are also capable of reacting with phenylglyoxal. No quantitative experiments were attempted to show whether the polypeptides plus diamino-acids could account for the whole of the reaction obtained with pancreatic extract, or whether some further substances have to be taken into account.

That the antiglyoxalase action of pancreatic extracts cannot be due to removal of the glyoxal by the reaction described in this paper, as suggested by Foster, seems to follow from a number of results described in the literature. Neither yeast nor testis extracts show any antiglyoxalase activity (Dakin and Dudley). Both Ariyama and Kuhn and Heckscher observed antiglyoxalase action while following methylglyoxal disappearance and not, like Dakin and Dudley or Foster, mandelic acid production. Finally the complete thermostability and easy dialysability of the substances responsible for the reaction here described disagree with the properties ascribed to antiglyoxalase by the above workers. The problem is being further investigated.

SUMMARY.

1. A method is described for following acid production from phenylglyoxal by means of the Barcroft manometer.

2. A production of acid, similar at first sight to that obtained with phenylglyoxal and liver extract, is obtained when phenylglyoxal is added to a pancreatic extract.

3. It is shown that this acid production is not due to a transformation into mandelic acid, but to a reaction with amino-compounds, partly diamino-acids, with formation of a sparingly soluble orange substance. The reaction seems to be identical with that described by Foster.

4. The diamino-acids present in pancreatic extract cannot account for the whole of the reaction obtained, but it is found that polypeptides, including those of monoamino-acids, also react, while the monoamino-acids themselves do not.

My thanks are due to Mr J. B. S. Haldane for suggesting the subject of this research, and to him and Sir F. G. Hopkins for their interest and encouragement.

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LIV. THE ALLEGED CONTAMINATION OF CAROTENE BY VITAMIN A.

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(*Received March 4th, 1930.*)

The claim of Euler, Euler and Hellstrøm [1928] that carotene can restore growth in rats receiving diets deficient in vitamin A has now been supported by various workers [Moore, 1929, 1, 2; Collison, Hume, Smedley-MacLean and Smith, 1929; Kawakami and Kimm, 1929]. Negative results, however, were obtained by Dulière, Morton and Drummond [1929, 1, 2], and it was suggested by these workers that pure carotene was inactive, and that samples found to be active must have contained traces of vitamin A as impurity. Recently Hume and Smedley-MacLean [1930] have shown that the real cause of this failure did not lie in the superior purity of the carotene used, but was in all probability traceable to the use of ethyl oleate as a solvent for the test doses. Nevertheless the criticism that even the purest samples of carotene might still contain traces of the familiar vitamin A of cod-liver oil has remained incompletely answered.

In the present note this problem has been approached by a comparison of the ultra-violet absorption spectra of carotene and cod-liver oil concentrate. It is well established that cod-liver oil and its concentrates are characterised by an absorption band at $328\mu\mu$, and this band is attributed with some confidence to the presence of vitamin A [Takahashi *et al.* 1925; Morton and Heilbron, 1928; Drummond and Morton, 1929]. In an examination of carotene samples of varying degrees of purity, however, Dulière, Morton and Drummond have failed to detect this band, and have suggested that the absorption of carotene in this region may be sufficiently dense to obscure completely any maximum due to the vitamin. From the experiments to be described below a different conclusion has been reached, and it will be shown that the activity of carotene cannot be correlated with sufficiently intense absorption at $328\mu\mu$ to account for its physiological activity on the basis of the presence as an impurity of the same factor as is responsible for the absorption band of cod-liver oil.

EXPERIMENTAL.

The carotene used was prepared from carrots by Dr T. Moore, and had been purified to M.P. 178° (uncor.) by 12 recrystallisations from cyclohexane. In biological experiments the minimal daily dose necessary to promote growth in rats had been found to be about 0·002 mg. The cod-liver oil concentrate was also supplied by Dr Moore, and had been found to promote growth regularly at 0·01 mg., with one isolated response at 0·0033 mg.

The solutions were made up in dry ether and examined in 1 cm. cells in a Hilger sector photometer used in conjunction with a Hilger E_3 quartz spectrograph, a condensed copper spark being employed as a light source. The absorption curves obtained are shown in Fig. 1.

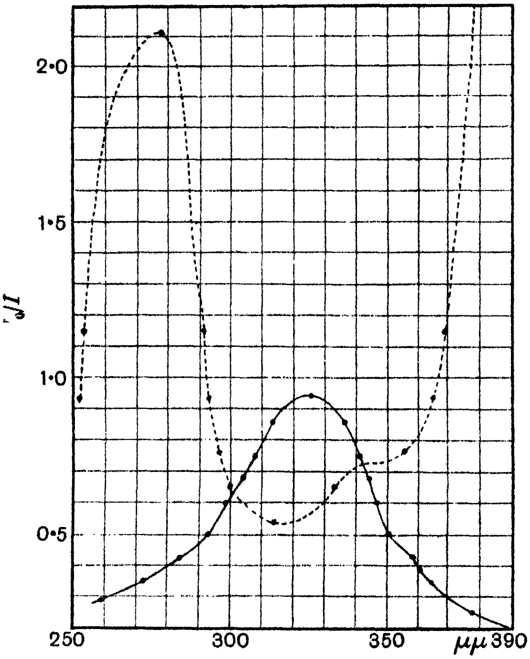

Fig. 1. Full curve: 0·005 % cod-liver oil concentrate.
Broken curve: 0·006 % carotene.

Discussion.

From Fig. 1 it will be seen that the absorption of carotene at 328 μμ was considerably less than the absorption of the cod-liver oil concentrate dissolved at the same concentration. Now since the carotene, weight for weight, had been found to be rather more active than the concentrate it would have been expected, if the vitamin A factor of the pigment were present in the same form as in cod-liver oil, that the absorption at 328 μμ would at least be equal in intensity, even in the improbable event of pure carotene being perfectly transparent in this region. Indeed if the carotene were much less active than was found, a maximum should still be apparent from the intensity of the band shown by the concentrate.

It is, therefore, obvious that the biological activity of carotene cannot be explained by the presence of vitamin A as characterised by a band of 328 μμ. Unless it be assumed that the close association between this band and the vitamin A of cod-liver oil is purely fortuitous it seems necessary to assume that vitamin A can exist in at least two forms, only one of which absorbs strongly at 328 $\mu\mu$ or that vitamin A can be synthesised *in vivo* from carotene, as has been suggested by Moore [1929, 3], or from some impurity still associated with the carotene.

SUMMARY.

1. The intense vitamin A activity of carotene is not accompanied by the absorption band at 328 $\mu\mu$ which is considered characteristic of the vitamin A of cod-liver oils.

2. The absorption of carotene (M.P. 178°) in this region is insufficiently intense to obscure the absorption at 328 $\mu\mu$ which would be entailed by the presence of the cod-liver oil factor in an amount sufficient to account for the physiological activity of the pigment.

3. Unless the close relation between the vitamin A activity of cod-liver oil and the 328 $\mu\mu$ band is deceptive it is essential to assume that vitamin A can exist in at least two forms, or that carotene behaves as provitamin A.

My best thanks are due to Dr Moore for suggesting this problem, and to Dr J. K. Marsh for his interest and help.

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LV. THE ISOLATION OF *l*-THYROXINE FROM THE THYROID GLAND BY THE ACTION OF PROTEOLYTIC ENZYMES.

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(*Received March 4th, 1930.*)

In previous communications by one of us [Harington, 1926, 1928] reference has been made to experiments with the object of isolating thyroxine from the thyroid gland by means of the action of enzymes alone. All these attempts had hitherto proved abortive, but the problem remained of such interest that it was determined to make a renewed attack upon it in a more systematic manner and upon a larger scale than had so far been attempted.

The problem seemed to us to present three main points of interest; in the first place the isolation of thyroxine through the unaided action of proteolytic enzymes would supply the final proof that the compound was present in the gland in peptide combination, provided that the possible objection that the isolation was rendered possible by the digestion of protein containing the thyroxine either in simple admixture or adsorbed could be dealt with on analytical grounds. Secondly, during the isolation of thyroxine by the action of enzymes alone no racemisation was to be anticipated, so that the determination of the optical rotation of the product would provide us with the information which we required as to the completeness of the resolution of *dl*-thyroxine already described [Harington, 1928]. In the third place the physiological investigation of the intermediate thyroxine-containing digestion products of the thyroid offered an opportunity of testing the correctness of the suggestion recently advanced [Harington and Randall, 1929] that any remaining discrepancy which appears to exist between the physiological activity of thyroid gland and that of an equivalent amount of thyroxine may be accounted for by an enhancement of the activity of the latter compound when it is in peptide combination.

One of the main difficulties which we anticipated was the loss of iodine as iodide which has been reported to occur in attempts to digest the thyroid protein with trypsin. Oswald [1908] has stated that in one experiment the whole of the iodine of a preparation of iodothyreoglobulin was thus liberated during digestion with an extract of pancreas for 3 weeks, and he has also published experiments [1909] to show that a similar liberation of iodine occurs when 3 : 5-diiodotyrosine is subjected to the action of trypsin. One of us [Harington, 1926] in the course of his earlier experiments on the isolation of thyroxine met with the phenomenon under discussion, although the liberation of iodine did not appear to be so extensive as was indicated by the results of Oswald.

As a preliminary step, therefore, we undertook some experiments concerning the action of various preparations of trypsin on diiodotyrosine, since the possibility was present to our minds that the liberation of iodine might not be due to the trypsin as such, but to some accompanying enzyme; we hoped in fact that it might prove possible to procure or to make a preparation of trypsin which, whilst retaining its proteolytic activity, would be free from the disadvantage that it also liberated iodine from compounds of this type.

To our surprise, however, with none of the preparations of trypsin which were at our disposal were we able to repeat Oswald's experiments. After 2–3 weeks of exposure to the action of trypsin under the conditions specified by him there was scarcely a trace of the effect which he describes, and, although on keeping the solutions for a period of months there was detectable a certain amount of liberation of iodine as iodide, this amount remained small, and we are inclined to ascribe the phenomenon to the effect of prolonged keeping in slightly alkaline solution, rather than to any action of the enzyme. We found, however, in the course of our experiments, that the technique of analysis was not so simple as at first appears. In the presence of nitric acid and silver nitrate even in the cold, the iodine of diiodotyrosine is liberated with comparative ease, and precipitated as silver iodide. In the experiments under discussion samples of the solutions were taken from time to time, and the state of affairs determined by treatment with nitric acid and silver nitrate to remove inorganic iodide, followed by filtration and determination of the organic iodine remaining in the filtrate. At times the filtration of the precipitate produced by silver nitrate in acid solution would be slow, and it was noticed that in such experiments the liberation of iodine appeared to have been greater; it was then found that by keeping the solution for different periods of time at the stage of precipitation with silver nitrate and nitric acid the result of the analysis could be varied almost at will. It is evident, therefore, that unless the analysis be carried out with the utmost care and rapidity the results obtained are of little value, and it seems to us not unlikely that the findings of the earlier investigator may be ascribed to an unsuspected error of technique of this description.

These experiments at any rate relieved us of a large part of our fear that the action of trypsin would be inevitably associated with serious losses of material through liberation of iodine, so long as the duration of the experiment were not unduly great. We could not indeed be certain that the conditions would be the same during the actual hydrolysis of a protein containing thyroxine (or diiodotyrosine) in peptide combination, or in the presence of oxidases which might be contained in the gland itself; there was, however, no indication of the necessity of attempts to purify the proteolytic enzyme further, and it was decided to begin work on the enzymic hydrolysis of the thyroid protein, the general plan being to make the enzymic attack of as intensive a nature and of as short duration as possible.

About 30 years ago Hutchison [1896, 1898] described experiments in which he was able, as the result of the action of pepsin on "colloid" (presumably crude iodothyreoglobulin) to obtain an acid-insoluble precipitate containing a considerably higher concentration of iodine than the starting material. The preparation of this product of peptic digestion appeared to us to offer a promising starting point for our experiments, more especially as preliminary peptic digestion is known to lay a protein more open to subsequent attack by trypsin.

As a source of material we have used, throughout the greater part of this work, chilled thyroid glands. These were obtained for us by the British Drug Houses in sealed tins, and were conveyed direct from the cold store to the National Institute for Medical Research, where, by the courtesy of Dr H. H. Dale, F.R.S., they were stored at – 5°. The material was taken from the cold store as required, rapidly minced, and extracted with a slightly alkaline solution containing 1 % of sodium chloride; the extract, which contained practically the whole of the iodine, was either adjusted to p_H 1·5 and submitted directly to intensive peptic digestion, or the globulin was first separated by dilution or by heat-coagulation, and a suspension of the precipitate in dilute hydrochloric acid at p_H 1·5 was digested with pepsin. In the latter case the action of the pepsin brought about rapid solution of the greater part of the precipitate, and in all cases after 48–96 hours the peptic action was complete. At this stage, the reaction of the solution having been maintained at p_H 1·5 by periodic additions of hydrochloric acid, the amount of precipitate was very small; on reducing the acidity, however, a considerable increase in precipitation occurred, and indeed a sharp optimum precipitation point was observed at p_H 5·0. The precipitate so obtained was large in amount and contained about 40 % of the total iodine at a concentration of about 1 % of the dry weight; it was, however, heavily contaminated with fat. Attempts to remove the fat at this stage were unsuccessful owing to the almost permanent emulsions which were formed with ether or other fat solvents. The material was therefore dissolved as completely as possible in dilute sodium hydroxide, the reaction being finally adjusted to p_H 8·0, and was subjected to digestion with trypsin. In spite of our previously mentioned failure to observe any liberation of iodine as the result of the action of trypsin on diiodotyrosine, we had nevertheless in mind, for reasons already mentioned, the possibility that during the actual digestion of the protein conditions might be different. We therefore conducted the digestion as intensively as possible, employing large amounts of an active preparation of trypsin and arresting the reaction as soon as the curve representing the increase of amino-nitrogen began to flatten out. This occurred after 72–96 hours, at which point the amino-nitrogen represented about 50 % of the total; the solution was diluted with water and adjusted to p_H 5·0 and the precipitate separated by filtration. It was found that at this stage a considerable amount of material of negligible iodine content could be rejected by extracting the precipitate with 70 % acetone containing some hydrochloric acid, nearly all the iodine passing into solution in this solvent and the larger part of the material being left undissolved. By adjustment of the reaction of the filtrate to p_H 5·0 and removal of the acetone by distillation the iodine-containing material was recovered. A fairly satisfactory removal of lipoids could now be effected by solution of the precipitate in dilute alkali followed by reprecipitation with simultaneous extraction with ether, and the residual material could be readily dried to a dark brown powder which usually contained about 15 % of iodine and 7 % of nitrogen.

It was resolved at this point to attempt to make use of the fact observed by Kendall [1919] that the pigmented substances of high molecular weight which are formed during the hydrolysis of the thyroid have a very low solubility in barium hydroxide. We therefore dissolved our material in a large volume of boiling water with the aid of the minimal amount of ammonia, and added barium hydroxide (hydrate) to make a concentration of 5 % of the latter; this produced a bulky precipitate, which was immediately filtered off from the hot solution; the filtrate, which was of a light brown colour, was adjusted to p_H 5·0 with hydrochloric acid. This caused an immediate precipitation, and further separation of material occurred on standing; after 48 hours the solid material was filtered off and dried, when it formed a light brown-coloured powder containing usually a little over 30 % of iodine and giving a very strong nitrous acid reaction for thyroxine.

Attempts to crystallise this material by dissolving it in 80 % alcohol containing sodium hydroxide and acidifying the hot solution with acetic acid resulted in no separation. When acetone was added to the acid solution, however, separation of a considerable amount of pigmented oily material of low iodine content occurred; after removal of the latter the clear liquid was poured into a large volume of water whereupon there slowly separated a cream-coloured product which, after drying, had an iodine content of 45–50 %, and a nitrogen content of about 5 %; since, as the result of a number of experiments, we arrived constantly at a product of approximately the same composition, in spite of the complexity of the process, we were at first inclined to believe that this substance, which, from the analytical figures, appeared to be a tri- or tetra-peptide containing thyroxine, represented a true end-product of tryptic digestion. It was possible to isolate thyroxine after hydrolysis of the material with a mixture of hydrochloric and acetic acids or with alkali, but we were unable to effect any hydrolysis by means of active erepsin preparations. In solubilities the substance differed markedly from thyroxine. Whereas thyroxine is practically insoluble in acid, the digestion product was precipitated from its alkaline solution sharply and completely on adjustment of the reaction to p_H 5·0 but only incompletely on addition of excess of acid. It was soluble in glacial acetic acid and partly so in 50 % acetic acid, particularly when hot. It failed to separate on addition of acetic acid to its solution in alkaline 80 % alcohol. In 0·1 *N* sodium carbonate the material was moderately soluble, but when a hot concentrated solution was allowed to cool slowly a separation of a colourless sodium salt occurred; the latter was, however, not crystalline and did not have the characteristic appearance of the sodium salt of thyroxine.

Table I. *Course of purification.*

Yields and atomic nitrogen : iodine ratios at various stages.

Starting material	Peptic product	After trypsin and ether	After baryta	After acetone-alcohol purification
A. Iodothyreoglobulin 2·17 g. iodine	0·95 g. iodine N : I 49	0·2 g. iodine N : I 3·4	—	—
B. Iodothyreoglobulin 1·455 g. iodine	0·88 g. iodine N : I 57	0·34 g. iodine N : I 6·3	0·071 g. iodine N : I 1·8	—
C. Iodothyreoglobulin 1·81 g. iodine	0·91 g. iodine N : I 60	0·30 g. iodine N : I 5·3	0·068 g. iodine N : I 2·5	—
D. Iodothyreoglobulin 4·65 g. iodine	Not analysed	0·85 g. iodine N : I 4·4	0·42 g. iodine N : I 1·9	0·145 g. iodine N : I 1·0
E. Desiccated thyroid 3·34 g. iodine	1·44 g. iodine N : I 55	0·65 g. iodine N : I 4·2	0·4 g. iodine N : I 1·0	0·17 g. iodine N : I 0·8
F. Desiccated thyroid 3·77 g. iodine	1·78 g. iodine N : I 76	1·07 g. iodine N : I 6·0	0·57 g. iodine N : I 1·3	0·25 g. iodine N : I 1·15
G. Iodothyreoglobulin 11·3 g. iodine	4·8 g. iodine N : I 57	(*a*) 1·15 g. iodine N : I 5·6 (*b*) 0·78 g. iodine N : I 6·5	(*a*) 0·47 g. iodine N : I 1·9 (*b*) 0·31 g. iodine N : I 2·8	0·32 g. iodine N : I 1·1
H. Iodothyreoglobulin 11·0 g. iodine	8·2 g. iodine N : I 109	1·7 g. iodine N : I 11·4	0·9 g. iodine N : I 2·2	(After 2nd trypsin) (*a*) 0·22 g. iodine N : I 0·98 (*b*) 0·45 g. iodine N : I 1·1
K. Iodothyreoglobulin 2·06 g. iodine	No pepsin	0·2 g. iodine N : I 4·3	0·075 g. iodine N : I 1·3	0·028 g. iodine N : I 0·6
L. Iodothyreoglobulin 12·6 g. iodine	No pepsin	2·4 g. iodine N : I 4·1	1·5 g. iodine N : I 1·0	0·63 g. iodine N : I 0·6

Much time was spent in attempts to separate a pure peptide from this digestion product, until the experiments described in detail in the experimental part led to the isolation from it, as the result of simple methods of purification, of a small amount of crystalline thyroxine. The character of the digestion product was thus revealed in its true light as a mixture of free thyroxine with a preponderance of thyroxine still in peptide combination.

In Table I are given figures for the yields of iodine and for the atomic nitrogen : iodine ratios of the thyroxine-containing digestion product at various stages of three incomplete and seven complete preparations carried out by the method described above. The nitrogen : iodine ratios give a useful indication of the extent of digestion at the various stages, and it will be seen that, except in preparation H where the early stages of the digestion were peculiarly inefficient, the figures show a remarkable degree of constancy. If we multiply these ratios by 4 we obtain the nitrogen : thyroxine ratios, and it may then be seen that the product after trypsin and ether treatment has the approximate composition of a thyroxine peptide containing 19 other amino-acids: after purification with baryta the product gives approximate figures for a hepta-peptide, and after the acetone-alcohol purification for a tri- or tetra-peptide containing thyroxine. All these deductions are naturally based on the assumption that no diamino-acids are present; the markedly acidic properties of the product indicate that this assumption, for the more highly purified material at least, is probably a fair one.

Table II shows the percentage of iodine in the thyroxine-containing digestion product at three significant stages of purification in the various preparations.

Table II.

Percentage of iodine in thyroxine-containing fraction at various stages of digestion and purification.

Preparation	After trypsin and ether	After baryta purification	After acetone-alcohol purification
A	15·3	—	—
B	—	29·1	—
C	2·2*	23·5	—
D	15·0	32·4	42·0
E	17·1	41·7	47·6
F	12·4	40·6	49·8
G	(*a*) 13·8	(*a*) 32·0	43·0
	(*b*) 11·9	(*b*) 29·0	
H	8·1	30·2	(*a*) 45·3†
			(*b*) 42·0†
K	15·7	38·1	49·8
L	10·2	43·8	56·5

* No ether. † After 2nd tryptic digestion.

In Table III are recorded the figures for the percentage of iodine and of total and amino-nitrogen in the more highly purified fractions of two preparations. The figures to which we wish to call special attention here are those in the last column, which represent the ratio of amino-nitrogen to thyroxine in the product. The determinations of amino-nitrogen were made by the colorimetric method of Folin [1922] on small amounts of material, and no very high degree of accuracy is claimed for them; it is, however, sufficiently obvious that the ratio approximates to unity; that is to say, that there is in the product one free amino-group for every molecule of thyroxine. Such an analytical result is consistent with the supposition that the material is composed exclusively of peptides containing thyroxine, or of thyroxine mixed with thyroxine-containing peptides; it could not, however, be obtained from a mixture of free thyroxine with peptide material of a different character. As collateral evidence in favour of the hypothesis that thyroxine is present in the thyroid in actual combination these analyses are therefore of value. More direct evidence in this direction is afforded by the fact that on prolonged acid hydrolysis of the digestion product, practically the whole of the nitrogen was obtained in the amino-condition. It appears therefore that there is no longer any room for doubt that thyroxine does exist in the thyroid gland in peptide combination—as a constituent amino-acid, in fact, of the characteristic thyroid protein, iodothyreoglobulin.

Table III.

Detailed analysis of partly purified digestion products.

Material		Iodine %	Total nitrogen %	Amino-nitrogen %	$\frac{\text{Amino-nitrogen}}{\text{thyroxine}}$
Preparation H. After baryta purification		30·2	7·42	0·97	1·2
Preparation H. After acetone purification	(*a*)	45·3	4·92	1·26	1·01
Ditto	(*b*)	42·0	5·2	1·01	0·88
Preparation K. After baryta purification		38·1	4·8	1·3	1·3
Preparation K. After acetone purification		49·8	3·4	1·34	0·97

The amount of crystalline thyroxine which could be separated from the digestion product was very small, and since the digestion product itself, as the result of large losses in the course of preparation, represented only a minute fraction of the original iodine, it was not possible to obtain more than the smallest amounts of thyroxine by this method. Sufficient was, however, isolated to permit of satisfactory determinations of optical activity with the aid of a Fischer micropolarimeter tube; these determinations indicated that the thyroxine was laevorotatory and had in fact a specific rotation of approximately $[\alpha]_{5461} - 3{\cdot}8^\circ$ as the sodium salt in 60 % alcoholic solution; this figure is in close agreement with that obtained for the *l*-thyroxine previously prepared [Harington, 1928] by the resolution of *dl*-3 : 5-diiodothyronine and subsequent iodination of the product. The resolution at that time described must therefore be assumed to have been moderately complete, and the suggestion, made on the basis of the relative physiological activity of the two enantiomorphs, that *l*-thyroxine would prove to be the naturally occurring variety, is confirmed. At the same time this result indicates, in view of the observations of Gaddum [1930] regarding the relative physiological activity of the two enantiomorphs, that no very great discrepancy between the activity of thyroid gland and that of thyroxine isolated therefrom by alkaline hydrolysis can be accounted for by racemisation alone.

This brings us back to the third point of interest of the present work, namely the physiological comparison of the digestion product, containing thyroxine for the most part still in peptide combination and containing no iodine compound other than thyroxine, with free thyroxine itself. This comparison was kindly carried out for us by Dr J. H. Gaddum. The substances were tested on rats and their effects were compared after administration both by the oral and the subcutaneous route. The oral administration of 1 mg. *per diem* for 6 days of pure *l*-thyroxine had practically no effect on the oxygen consumption of a rat, whilst an equivalent dose of the digestion product (1·3 mg. *per diem* for 6 days of a preparation containing 50 % of iodine) produced an increase in the oxygen consumption of 85 %. By the subcutaneous route, on the other hand, single doses of 1 mg. of *l*-thyroxine and 1·3 mg. of the digestion product caused increases in the oxygen consumption of 32·5 % and 37·5 % respectively; that is to say, in this case the physiological difference between the two substances was very much less marked.

This experiment appears to us to afford an admirable demonstration of the causes which have led various workers to form what we believe to be a false conclusion, namely that the physiological activity of the thyroid gland cannot be entirely accounted for by the thyroxine which it contains. It is indeed quite unjustifiable to deduce any such discrepancy from an apparent difference between the effects of thyroxine and thyroid gland when given by mouth or otherwise. The physical properties of thyroxine are such as to make it highly probable that the absorption of this substance after oral administration would be inefficient and erratic; the digestion product, on the other hand, possessing as it does a much wider range of solubility, might well be absorbed almost quantitatively; such a state of affairs is precisely what is indicated by the first part of the experiment of Dr Gaddum which has just been described. For a similar reason it is equally unjustifiable to attempt to make a comparison between the effect of thyroxine and that of thyroid gland or iodothyreoglobulin when both are injected subcutaneously. In view of the slowness with which the physiological effect of thyroxine develops, it is almost a foregone conclusion that after an injection of a massive dose of the substance a portion will be lost by excretion before it has time to exercise its action on the organism. On the other hand, it is known that the processes involving the removal of a foreign protein from the body after its subcutaneous injection may take a number of days; after the subcutaneous injection of iodothyreoglobulin therefore we should anticipate a more or less prolonged period during which the active principle of the protein would be slowly liberated, thus attaining a therapeutic efficiency greatly in advance of that achieved by injection of a single equivalent dose of free thyroxine. In the last sentence we have used the term "active principle" of the iodothyreoglobulin rather than thyroxine because it appears to us to be probable that the actual substance which the thyroid secretes into the blood may not be free thyroxine but a relatively simple peptide containing this compound—a substance in fact analogous to our final digestion product. This conclusion is supported by the results of recent experiments of Dr Reid Hunt in Boston in which a sample of the digestion product containing approximately 50 % of iodine, from which as much free thyroxine as possible had already been separated, has shown itself to be certainly 250 and possibly even 500 times as active as U.S.P. standardised desiccated thyroid, both substances being administered orally. The digestion product when administered intravenously was approximately as active as when given by mouth. These results were obtained in mice by the acetonitrile method. Dr J. H. Means of Boston has also studied the calorigenic action of this digestion product when given by mouth to patients with a sub-normal basal metabolic rate; although his controls, which consist in observing the effect of U.S.P. standardised thyroid in the same patients, have not yet been completed, it is already apparent that the digestion product is fully as effective as an equivalent dose of thyroid and very likely more so. The U.S.P. standardised thyroid contains about 0·2 % of iodine, so that the results which have just been quoted mean that, in order to produce a given physiological effect, at least as much and probably more iodine must be given in the form of thyroid than in the form of the digestion product which contains no iodine compound other than thyroxine. This is precisely what our recently acquired knowledge of the chemistry of the thyroid would lead us to expect. There remains, therefore, in our opinion no reason whatever to presume the existence in the thyroid gland of an active principle other than thyroxine, or that the thyroxine in the thyroid exists in an "activated" form except in so far as owing to its optical activity and possibly also to the fact that it is in peptide combination, it can produce an effect greater than that of the free racemic compound. Indeed, taking into account the errors of the physiological method and the essential difficulties of obtaining a therapeutic comparison to which we have called attention, it is a matter for surprise that even an approximate parallelism can be observed between the action of thyroid gland or iodothyreoglobulin or an equivalent amount of free *l*-thyroxine.

EXPERIMENTAL.

A. *Action of trypsin on diiodotyrosine.*

These experiments were undertaken to test the statements of Oswald regarding the hydrolysis of the iodine of diiodotyrosine by trypsin, and to obtain an indication of the extent to which this reaction was likely to prove a serious obstacle in the way of the isolation of thyroxine by enzymic action.

A solution of diiodotyrosine (prepared from *l*-tyrosine) containing 0·5 mg. of iodine per cc. was buffered with phosphate and adjusted to p_H 8·0; 100 cc. samples of this solution were treated with active preparations of trypsin (two commercial samples and one fresh pancreatic extract), toluene being added to inhibit bacterial action, and were placed in an incubator at 40°. Portions of the solutions were withdrawn at intervals and analysed as follows: cold boiled-out nitric acid and silver nitrate were added, and, after 15 minutes, the solution was filtered through asbestos; the filtrate and washings were freed from silver by addition of hydrochloric acid; after removal of silver chloride the solution was made alkaline, evaporated, and analysed for iodine by the method of Kendall [1914]; the figure obtained was taken to represent the iodine remaining in organic combination, any iodide which had been formed having been removed by the preliminary treatment with silver nitrate and nitric acid.

Results of analyses carried out in this manner indicated no significant alteration in the organic iodine after 1, 2 and 3 weeks' digestion respectively. The solutions were then left undisturbed for 5 months and again analysed. The organic iodine was now found to be diminished to about 80 % of the total iodine, but this diminution was just as marked in the control solution, to which no enzyme had been added, as in those containing active trypsin. It was evident, therefore, that no liberation of iodine as iodide could be ascribed to the action of the trypsin as such. In the effort to account for the discrepancy between our results and those of Oswald, we studied the conditions of the analysis more closely, and found, in fact, that increase either in the amount of nitric acid employed (even though the latter were freshly boiled out) or in the length of time allowed for the precipitation in presence of nitric acid, caused an apparent diminution in the proportion of organic iodine. It is known that the iodine of diiodotyrosine is removed with extreme ease when the compound is warmed with silver nitrate and nitric acid; there is little doubt in our minds but that this reaction takes place also to some extent in the cold, and that an error of this type accounts at least in part for the results obtained by Oswald. There exists the further possibility that the enzyme preparations employed by him contained oxidases which were absent in our experiments, and which may have brought about some destruction of the diiodotyrosine.

B. *Enzymic hydrolysis of thyroid gland.*

The early stages of the digestion (leading to the material containing about 15 % of iodine) were carried out by a variety of methods, of which typical examples are described.

(*a*) *Successive action of pepsin and trypsin on iodothyreoglobulin.* Frozen thyroid gland (5400 g.) was passed rapidly through a mincer and suspended in 5400 cc. of 0·02 % sodium hydroxide containing 1 % of sodium chloride; the suspension was shaken for 3 hours and allowed to stand overnight; it was then strained through butter-muslin and the solid residue was submitted to a second similar extraction. The combined reddish-coloured extracts were chilled and as much fat as possible was removed mechanically. The solution was adjusted to p_H 5·0, treated with sodium sulphate to make 1 % concentration of the latter, and raised rapidly to boiling-point by introduction of a vigorous current of steam with simultaneous external heating. The coagulum of crude iodothyreoglobulin, which contained about 97 % of the total iodine of the extract, was filtered off hot through large Büchner funnels, and the cheesy cakes of material so obtained were suspended in 10 litres of water containing 0·5 % of an active pepsin preparation and sufficient concentrated hydrochloric acid (in this case 240 cc.) to bring the reaction of the solution to p_H 1·6. The mixture was transferred to large bottles which were placed in an incubator at 40°, the contents being frequently shaken during the early stages of the digestion. After 48 hours fresh pepsin (the same amount as originally employed) was added, the reaction being adjusted to p_H 1·6 by the further addition of hydrochloric acid if necessary. After a further 48 hours the solution was diluted with an equal volume of water and the reaction was adjusted by the cautious addition of sodium hydroxide, vigorous mechanical stirring being employed to obtain efficient admixture; at p_H 5·0 a sharp flocculation was obtained. After standing for a few hours the mixture was poured on to large pleated filters and allowed to drain; filtration was slow and no pretence at efficient washing could be made. The bulky greasy precipitate, containing about 1 % of iodine reckoned on the dry weight and about 40 % of the total iodine of the iodothyreoglobulin, was scraped off the filters and suspended in water (2370 cc.); sodium hydroxide was added until the reaction was p_H 8·0 and the greater part of the material had passed into solution; 0·2 % of an active preparation of trypsin was then added and the material was transferred to the incubator. The reaction was readjusted to p_H 8·0 twice daily by addition of the necessary amount of sodium hydroxide; daily determinations of the amino-nitrogen concentration were made by the Van Slyke method. After 72 hours a second addition of 0·2 % of trypsin was made. At the end of 96 hours from the beginning of the digestion, the rate of increase of the amino-nitrogen (which had by this time reached about 50 % of the total nitrogen) had become very low; as we were anxious so far as possible to avoid the chance of loss of iodine, the digestion was interrupted at this stage by bringing the solution to p_H 5·0 by the addition of hydrochloric acid. The precipitate was again filtered off on pleated filters and washed as well as possible with water; it was then extracted three times in succession with 70 % acetone to which was added enough hydrochloric acid to keep the reaction about p_H 1·6; the bulky insoluble matter was filtered off and the filtrates were immediately brought back to p_H 5·0 with sodium hydroxide. The acetone was then removed by distillation, diminished pressure being used in the last stages. The residual aqueous liquid contained a dark-coloured precipitate of somewhat oily consistency; on addition of sodium hydroxide all passed into solution, and the alkaline liquor was transferred to a beaker and covered with a layer of ether. While the two layers were kept thoroughly mixed by vigorous mechanical stirring, hydrochloric acid was cautiously added until the p_H was again 5·0; the mixture was allowed to stand, when it separated into a brown aqueous layer and an almost black ethereal layer with a dark precipitate at the interface; the latter was filtered off and washed with ether, when it amounted to 5·66 g. of a dark brown powder containing 15·0 % of iodine and 7·28 % of nitrogen.

(*b*) *Successive action of pepsin and trypsin on alkaline sodium chloride extract of thyroid gland.* In some experiments the isolation of the crude iodothyreoglobulin by heat coagulation was omitted, and the solution obtained by extraction of the gland material with 0·02 % sodium hydroxide containing 1 % sodium chloride, after freeing as far as possible from fat, was adjusted to p_H 1·6 by addition of hydrochloric acid and submitted directly to peptic digestion, followed by the treatment described above. In one such experiment, 9·5 kg. of frozen thyroid gave 16 g. of material containing 13 % of iodine and 8·5 % of nitrogen.

(*c*) *Successive action of pepsin and trypsin on desiccated thyroid gland.* Desiccated and finely powdered thyroid gland (725 g.), which had been defatted with light petroleum, was suspended in 7600 cc. of water to which had been added 0·5 % of pepsin and 155 cc. of concentrated hydrochloric acid; the pepsin was renewed after 36 hours and the digestion was arrested at the end of 72 hours; the reaction of the solution was brought to p_H 5·0 and the resulting precipitate was submitted to tryptic digestion and worked up as described above; there was obtained 3·8 g. of material containing 17·1 % of iodine and 8·0 % of nitrogen.

(*d*) *Action of trypsin only on alkaline sodium chloride extract of thyroid gland.* The extract obtained in the usual manner from 7840 g. of frozen thyroid was submitted directly to digestion with 0·2 % of trypsin, toluene being added to inhibit bacterial action; the trypsin was renewed after 48 hours, the reaction being maintained at p_H 8·5 by periodic additions of sodium hydroxide and at the end of 76 hours the solution was brought to p_H 5·0; the precipitate was then extracted with 70 % acid acetone and the extracted material reprecipitated in presence of ether as already described; there was obtained a precipitate, a sample of which showed an iodine content of 8·3 % and nitrogen 7·8 %; this was subjected to a second similar tryptic digestion, the volume being at first 1200 cc. and being raised after 24 hours to 2200 cc.; after the second addition of trypsin the p_H of this digest, which had fallen spontaneously to 7·6, was allowed to remain at this figure for a day, with the intention of favouring the action of any dipeptidase which might be present in the enzyme preparation; the p_H was then raised to 8·0 again at which it remained unchanged despite the further addition of trypsin; after a total digestion period of 84 hours the material was again precipitated at p_H 5·0, and now contained 10·2 % of iodine and 4·7 % of nitrogen. As might be expected the ethereal layer in all the above-described experiments, being heavily loaded with lipoidal substances, carried a certain amount of iodine-containing material into solution; a large proportion of this separated out on keeping the solution. Thus in one experiment the ethereal layer contained, immediately after separation, 130 mg. of iodine; three weeks later a further precipitate had separated and the iodine content of the solution was 74 mg.; still more material continued to separate after this.

The digestion product obtained by any of the methods described above formed a dark brown or almost black powder which was readily soluble in alkali and much less so in acid; it had a well defined point of maximum flocculation at p_H 5·0; it contained usually 12–17 % of iodine and 5–8 % of nitrogen; that is to say, it had approximately four atoms of nitrogen for every one atom of iodine, or 16 nitrogen atoms for every molecule of thyroxine.

The next stage in the purification of the product was the removal of the pigmented impurities of relatively high molecular weight by making use of the insolubility of their barium salts. The conditions for this experiment were selected with a view to attainment of the maximum salting-out effect of the barium ions consistent with the exclusion of the possibility of hydrolysis. With this object the concentration of barium hydroxide was not raised above 5 % and the time of exposure even to this small concentration was cut down to a minimum. An example of the procedure may be given as follows. A sample of the tryptic digestion product containing 14 % of iodine and 8·5 % of nitrogen, and representing in all 1160 mg. of iodine, was dissolved in 1200 cc. of water with the aid of the minimum quantity of ammonia; the solution was brought to the boil and treated with 60 g. of crystalline barium hydroxide dissolved in 120 cc. of hot water; boiling was continued for 2 minutes, and the heavy precipitate was filtered off. Filtration was rapid, and the filtrate, whilst still hot, was acidified with concentrated hydrochloric acid; addition of excess of the latter caused partial re-solution of the precipitate; the reaction was therefore brought back to p_H 5·0 by the addition of sodium acetate. After standing for 48 hours at the ordinary temperature the precipitate was filtered off and dried. It contained 468 mg. of iodine at a concentration of 32 % and the nitrogen content was 6·3 %, corresponding to a ratio of 1·86 atoms of nitrogen to 1 atom of iodine. Although therefore the loss of iodine at this stage was heavy, the improvement in the quality of the product far outweighed this disadvantage. The product at this stage was a buff-coloured powder which gave the nitrous acid reaction for thyroxine with intensity. It was easily soluble in alkali, but insoluble in water at p_H 5·0 and only partly soluble at more acid reactions. When it was dissolved in 80 % alcohol with the aid of sodium hydroxide and when the solution was acidified with acetic acid no separation took place; acetone was added to such a solution in the hope of inducing the separation of any free thyroxine which might be present, and it was found that this treatment produced a precipitate of gummy material which contained relatively little iodine and much nitrogen. This observation led to the development of the next stage in the purification. Thus in one experiment 1·48 g. of material resulting from a treatment with barium hydroxide as already described, containing 40·7 % of iodine and 6·0 % of nitrogen (nitrogen : iodine = 1·34), was dissolved in 10 cc. of *N* sodium hydroxide and the solution was then made 80 % with respect to alcohol; a small precipitate was removed by centrifuging and the mother liquor was acidified by the addition of 4·5 cc. of 33 % acetic acid; 4 volumes of acetone were then added and the solution was rapidly centrifuged; the combined clear and light yellow mother liquor was diluted with 1500 cc. of water. Within a few minutes the opalescent solution began to deposit a light flocculent precipitate which gradually increased in amount. After 24 hours this was filtered off and amounted to 0·46 g. of material containing 49·8 % of iodine and 6·3 % of nitrogen; the nitrogen : iodine ratio was therefore 1·15 and the nitrogen : thyroxine ratio 4·6. After standing for

3 days longer a further crop of 71 mg. of material containing 46 % of iodine separated.

The fact that the product at this stage still exhibited marked differences in solubility from thyroxine, although, judging by the iodine content, it contained over 75 % of the latter, led us for a long time to suppose that we were dealing with a somewhat impure compound of thyroxine (probably a tripeptide) rather than with a mixture of free thyroxine with impurities; we were supported in this view by the constancy with which we arrived at a closely similar end-product in spite of the complexity of the preceding manipulation. Finding ourselves unable to achieve any further hydrolysis either by the aid of animal erepsin preparations of proved activity or with the help of yeast peptidases, we had recourse to acid hydrolysis of this product and also of material at an earlier stage of purification in the attempt to isolate thyroxine. We were in fact successful in obtaining thyroxine by hydrolysis with a mixture of hydrochloric and acetic acids at 100°, and we were able to show that during this process the whole of the nitrogen was converted into the amino-condition. Nevertheless the losses involved by the liberation of iodine were severe and, as will be shown later, the thyroxine obtained by this method was partly racemised.

Much effort was then expended in the attempt to obtain our supposed tripeptide in a pure condition, and it was during these experiments that we stumbled on the true explanation of the situation, namely that the purified digestion product consisted of a mixture of free thyroxine with thyroxine peptides.

The substance approximated more closely than the earlier products to thyroxine in that it had almost completely lost its solubility in excess of mineral acid. It still failed to separate, however, when acetic acid was added to its solution in alkaline 80 % alcohol. It was soluble in glacial acetic acid from which it could be precipitated with ether, without, however, effecting any significant purification; in 50 % acetic acid it could be dissolved by boiling and a part separated on cooling; on some occasions this less soluble portion seemed to show signs of commencing crystallisation, but a second separation from acetic acid failed to improve it to any significant extent, although fractions could be obtained in this manner which showed a somewhat higher iodine : nitrogen ratio. It was then decided to try and utilise the low degree of solubility of the product in cold 0·1 *N* sodium carbonate. This property, which has been found to be a general characteristic of the acidic compounds of the thyroxine series, appeared in the substance under discussion for the first time among the various products of digestion of the thyroid, the thyroxine-containing fraction at all earlier stages showing no tendency to separate from a concentrated solution in sodium carbonate. Very concentrated solutions of the material containing 45–50 % of iodine could be prepared in boiling 0·1 *N* sodium carbonate, and if such solutions were rapidly cooled they not infrequently formed stiff gels. By slow cooling, however, the separation of a sodium salt could be induced, and although the concentrated solution was rather dark in colour, the sodium salt, whilst not yet crystalline, was almost colourless. Moreover the material recovered after separation of the sodium salt on the centrifuge, and acidification of its solution in hot water, was definitely less soluble in acetic acid. A second separation from sodium carbonate at this stage involved heavy loss of material without corresponding improvement in quality; we therefore made use of the fact, which we had observed, that the substance could be precipitated from its solution in pyridine by dilution with water. A fairly concentrated solution in pyridine was prepared, and was diluted with boiling water to permanent turbidity; on cooling, a precipitate separated which showed a tendency to adhere to the walls of the tube where the latter was scratched with a glass rod, and which, under the high power of the microscope, showed commencing crystallisation; this material was separated on the centrifuge and dissolved in boiling 0·1 *N* potassium carbonate; the white precipitate which separated when the solution was kept for some hours at 0° was decomposed with acetic acid, and the product, after being washed with water on the centrifuge, was again dissolved in boiling dilute sodium carbonate solution; this time, as the solution cooled, precipitation of a heavy white microcrystalline substance occurred, which had the characteristic appearance of the mono-sodium salt of thyroxine. This salt was separated on the centrifuge, and dissolved in 80 % alcohol with the aid of sodium hydroxide; the boiling solution was acidified with 33 % acetic acid when rapid separation of typical crystals of thyroxine occurred.

The thyroxine so obtained melted at 235° with decomposition; this melting point was not changed by admixture with synthetic *l*-thyroxine which also melted at 235°. A mixture of synthetic *d*- and *l*-thyroxine sintered at 228° and melted at 231–232°.

The amount of thyroxine isolated from the digestion product was so small as to necessitate the use of a Fischer micropolarimeter tube for the determination of its specific rotation. A tube manufactured by Messrs Schmidt and Häntzsch was employed and considerable care was taken, by preliminary experimentation with known substances, to acquire a reliable technique. No difficulty was experienced in obtaining consistent readings, although there was a constant small difference between the readings of two observers. In order so far as possible to control this source of variation, determinations were made by the two observers on synthetic *l*-thyroxine as well as on the product obtained from the thyroid, the thyroxine in both cases being dissolved at approximately 5 % concentration in a mixture of 2 volumes of alcohol and 1 volume of *N* sodium hydroxide. The results are given in Table IV, from which it will be seen that, within the limits of error of the experiment, no difference could be detected between the synthetic and natural product; moreover, the mean of the four determinations is in close agreement with the value previously obtained, using a large polarimeter tube, for the synthetic *l*-thyroxine, viz. – 3·5°[1] [Harington, 1928].

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[1] Cf. note to Table IV.

Table IV.

Observer	Natural thyroxine ($c = 4{\cdot}4$) $[\alpha]_{5461}$	Synthetic *l*-thyroxine ($c = 4{\cdot}45$) $[\alpha]_{5461}$
C.R.H.	$-4{\cdot}45°$	$-4{\cdot}5°$ ($-3{\cdot}5°$)*
W.T.S.	$-3{\cdot}2°$	$-3{\cdot}15°$
Mean	$-3{\cdot}8°$	$-3{\cdot}8°$

* The results previously recorded [Harington, 1928], for the specific rotations of *d*- and *l*-thyroxine were erroneously calculated on the concentration by *weight* instead of by volume. The figure given here in brackets represents the earlier value corrected for the specific gravity of the solution in order to make it comparable with the present results.

Isolation of thyroxine by acid hydrolysis of digestion product.

Before we had realised the possibility of separating free thyroxine from the digestion product by ordinary methods of purification, we attempted to obtain optically active thyroxine by acid hydrolysis. The material was dissolved in 90 % acetic acid which was saturated with hydrogen chloride at 0°, and the mixture was heated on the steam-bath in a sealed tube for 6 hours. The solution was evaporated to dryness under diminished pressure and the residue was dissolved in 60 % alcohol with the aid of a little hydrochloric acid. Sodium hydroxide solution was added to bring the reaction to p_H 5·0, and, after standing overnight, the precipitate was filtered off, washed with water, and dissolved in the minimum amount of boiling 0·5 % sodium carbonate. The sodium salt of thyroxine, which separated on cooling, was converted into the free amino-acid in the usual manner. The thyroxine so obtained had $[\alpha]_{5461} - 1{\cdot}3°$ ($c = 4{\cdot}88$ in 60 % alcohol containing sodium hydroxide); considerable racemisation had therefore taken place. The hydrolysis was accompanied with some decomposition, free iodine being present at the end of the reaction and the yield of thyroxine was therefore poor. In the solution left after separation of the thyroxine, 66 % of the nitrogen was in the amino-condition; after further hydrolysis the proportion of amino-nitrogen was increased to 84 % of the total; there seems therefore no reason to doubt the peptide nature of the digestion product.

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LVI. THE PROPERTIES OF PURE GLUTATHIONE[1].

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The sulphur-containing peptide glutathione was first isolated by Hopkins [1921] in a non-crystalline form. He showed that it contained cysteine and glutamic acid, and the analytical figures seemed to indicate that it was a dipeptide containing those two amino-acids alone. Like cysteine itself, the substance in neutral aqueous solution rapidly absorbed oxygen, becoming converted into the oxidised or disulphide form. Hopkins showed that this disulphide form was rapidly reduced back to the sulphydryl form by animal tissues, so that it seemed clear that part of the oxygen normally absorbed by the respiring tissues was being utilised by the glutathione system.

While the nature of the tissue systems which reduce the disulphide group is not yet clear, the reduction is due in part, as Hopkins and Dixon [1922] showed, to factors which are not destroyed by heating the tissue to 100°. If for instance fresh muscle was minced, thoroughly extracted with water, heated to 100° and finally extracted with alcohol, it was found that the resulting "thermostable preparation" was still able vigorously to reduce oxidised glutathione. An aqueous suspension of such a preparation was unable to absorb oxygen, but on the addition of a little disulphide-glutathione (which of course takes up no oxygen by itself) an oxygen uptake was at once established. It was shown that the reduction of the disulphide group was due to "fixed —SH groups," *i.e.* sulphydryl groups, apparently forming part of the protein structure, which were not soluble in water and which were unable to react directly with oxygen (or methylene blue). These groups became oxidised by reducing the added disulphide, and the reduced glutathione formed then reacted with the oxygen.

The work of Warburg and Sakuma [1923] showed that the apparent autoxidation of cysteine was in fact due to small traces of catalytic metals (iron or copper) present as impurities. They showed that absolutely metal-free cysteine solutions absorbed oxygen only at a negligible rate, that the addition of a trace of ferrous salt to cysteine enormously accelerated its oxidation, and that its oxygen uptake was abolished by adding a little HCN, which inactivated the metals. These observations were confirmed and extended to glutathione by Harrison [1924], who showed that haematin compounds were also catalytically active. These facts did not, however, seriously affect the position as far as the biological action of glutathione was concerned, as in any case the tissues contained the iron necessary to make the glutathione autoxidisable.

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[1] A brief account of some of the main points of this work was communicated to *Nature*, Oct. 5, 1929.

The "thermostable system" was later studied in greater detail by Hopkins [1925], who showed that the total amount of disulphide which could be reduced per gram of tissue preparation was equivalent to the fixed —SH groups therein, as also was the total amount of methylene blue reduced by the tissue in presence of a little oxidised glutathione. The amount of oxygen taken up by the system was, however, much larger, amounting to several times the equivalent of the —SH groups originally present. The extra oxygen thus taken up was in fact utilised by the system for oxidation of the proteins present. The mechanism of this protein oxidation is not yet clear, but it appeared to be linked in some way with the oxidation of the sulphydryl group of glutathione, and it was thus dependent ultimately upon the fixed —SH groups, which maintained the glutathione in its reduced form. When these became exhausted all oxygen uptake ceased. Other proteins containing —SH groups (*e.g.* denatured egg-albumin) were also oxidised by glutathione, but proteins with no —SH groups (*e.g.* natural egg-albumin) were not.

Hopkins also showed that the sulphydryl form of glutathione (though not its disulphide form) strongly catalysed the autoxidation of unsaturated fats and fatty acids, particularly in acid solution. The phenomena observed were complex [Hopkins, 1925; Allott, 1927], and the mechanism of the catalysis is still unknown. It is clear, however, that the process is of a different nature from those described above, since the oxidation was shown not to depend upon the oxidation of the reduced glutathione, and indeed to occur equally well under conditions where glutathione is not autoxidisable. The system did not reduce methylene blue.

At this time glutathione was still thought to be a dipeptide of cysteine and glutamic acid. Later, however, Hunter and Eagles [1927] threw some doubt on this structure, and suggested that serine might also be a constituent of the molecule. Johnson and Voegtlin [1927] later published an apparent confirmation of the original view of Hopkins, who, however, reinvestigated the matter, and succeeded [Hopkins, 1929] in finding an elegant method for the ready isolation of glutathione in the pure crystalline state. He then found that the substance was a tripeptide containing cysteine, glycine and glutamic acid in equivalent amounts. Shortly afterwards Kendall, McKenzie and Mason [1929], working quite independently, also succeeded in obtaining crystalline glutathione, and showing that it was a tripeptide composed of those amino-acids.

This work showed that the earlier preparations of glutathione, with which all the work on oxidation catalysis had been carried out, were in fact impure, although they undoubtedly contained a large proportion of the tripeptide. The analytical figures make it probable that they contained free cysteine in addition. In view of these facts it was necessary that previous work on the catalysis of oxidations by glutathione should be reinvestigated with the pure crystalline material. The results of such an investigation are given in the present paper. It was soon evident that in several respects the behaviour of crystalline glutathione is very different from that of the earlier preparations, and the study of these differences has yielded results which we believe will be of importance in the elucidation of the glutathione problem.

EXPERIMENTAL.

The oxygen uptakes were measured in Barcroft manometers as modified by Dixon and Elliott (private communication). The total volume of liquid in each flask was always 3 cc. Any CO_2 formed was absorbed by KOH solution. Metal-free water and buffer solutions were used throughout, and the vessels were carefully freed from metals with acid and washed with pure water before use.

The crystalline glutathione was made from yeast by the method of Hopkins [1929]. Nitrogen and sulphur determinations corresponded excellently with the theoretical values for the tripeptide formula. Several different preparations were used, and all behaved in the same way.

The (lipin-free) thermostable tissue preparations were made by the method of Hopkins [1925].

Oxygen uptake of glutathione in aqueous solution.

The crystalline glutathione, like the older preparations, absorbs oxygen when shaken in air in phosphate buffer or water at p_H 7·6. The total amount of oxygen taken up corresponds with that calculated on the basis of the equation

$$4GSH + O_2 = 2GSSG + 2H_2O.$$

For example, in eight consistent determinations of total oxygen uptake under these conditions the mean value found was 99 % of the theoretical. The rate of absorption of oxygen at p_H 7·6 and 20° is about 30 $mm.^3$ per 5 mg. glutathione per hour. This is definitely lower than that of most of the older preparations, and varies much less from one preparation to another than was the case with them.

Its oxidation is strongly inhibited by *M*/1000 cyanide, and completely prevented by *M*/100 KCN. The oxidation is therefore presumably due to the presence of traces of catalytic metals, as in the case of cysteine.

There is, however, one respect in which crystalline glutathione differs completely in behaviour from cysteine (and from the earlier preparations of glutathione). Whereas the addition of a trace of iron or copper salt greatly accelerates the uptake of oxygen by cysteine or impure glutathione, the oxidation of crystalline glutathione is not accelerated at all by the addition of iron or copper at p_H 7·6, or by haematin in low concentration. Therefore the catalytic metal cannot be the limiting factor in the oxidation of the new tripeptide preparations. In Fig. 1 is shown the effect on the oxidation of old and new glutathione preparations of adding 0·001 mg. of iron. It will be seen that, whereas the rate of oxygen uptake in the first 20 minutes has been doubled by the metal in the case of the impure glutathione, that of the crystalline preparation is quite unaffected. The addition of this amount of iron to cysteine would, according to Warburg and Sakuma's [1923] figures, produce an additional uptake of roughly 100 mm.^{3} O_2 per hour, which is of the same order as the effect with the impure glutathione.

The absence of any accelerating effect with pure glutathione is the more remarkable in that quite large amounts of iron can be added without producing the slightest acceleration: the oxidation still proceeds at its original slow rate under conditions where the velocity of oxygen uptake by cysteine or impure glutathione becomes relatively enormous.

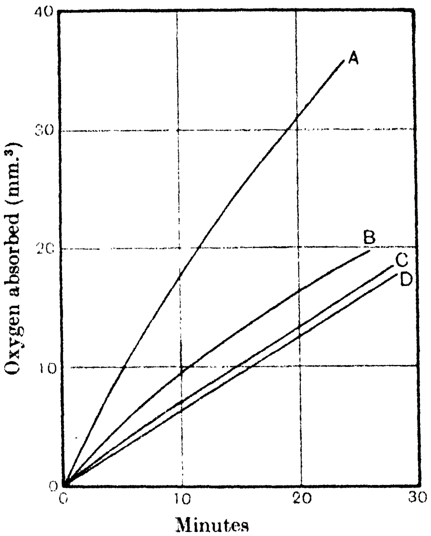

Fig. 1.

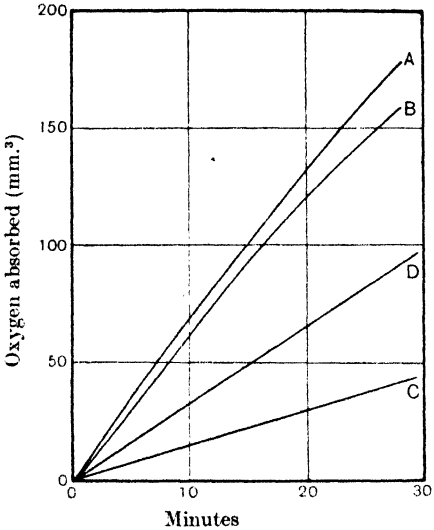

Fig. 2.

Fig. 1. Effect of iron on autoxidation of impure and crystalline glutathione. *A*, 5 mg. old glutathione + 0·001 mg. Fe; *B*, 5 mg. old glutathione; *C*, 5 mg. crystalline glutathione + 0·001 mg. Fe; *D*, 5 mg. crystalline glutathione. All in 3 cc. phosphate buffer p_H 7·6 at 18°.

Fig. 2. Effect of haematin on autoxidation of impure and crystalline glutathione. *A*, 10 mg. crystalline glutathione; *B*, 10 mg. crystalline glutathione + 0·05 mg. haematin; *C*, 10 mg. old glutathione. *D*, 10 mg. old glutathione + 0·05 mg. haematin. All in 3 cc. phosphate buffer p_H 7·6 at 40°. The sample of impure glutathione was different from that used for Fig. 1.

The behaviour with haematin is also interesting. Small concentrations of haematin behave like free iron, having no effect on the velocity of oxidation of the crystalline glutathione, but accelerating that of the impure preparations, as shown in Fig. 2.

With larger amounts of haematin, however, a definite acceleration is produced (as shown later in Fig. 13), although the catalytic activity is still small compared with that observed with cysteine. It is not yet quite clear how these results are to be interpreted.

A further point of difference between the crystalline and impure glutathione is that while the latter reduces haematin at a moderate rate, pure glutathione is unable to do so. In two Thunberg tubes were placed 5 cc. phosphate buffer (p_H 7·6) containing 0·1 mg. haematin and 5 mg. pure and impure glutathione respectively. The tubes were then evacuated and allowed to stand at room temperature. After 10 minutes the tube containing the impure glutathione was opened and examined with the spectroscope after the addition of pyridine. Strong absorption bands of pyridine-haemochromogen were observed, indicating that reduction of the haematin had taken place. After 30 minutes the other tube was examined in the same way, but no trace of haemochromogen could be detected, showing that the pure glutathione had been quite unable to reduce the haematin.

It is clear from the results previously given that although the oxidation of glutathione is due to catalysis by metals (and further evidence for this is given below) the slowness of the oxidation of the crystalline preparations is not due to any lack of catalytic metals, and the rate of oxidation must be determined by some other factor. The facts seem most readily explained by the assumption that the active catalyst is not the metal itself, but a complex of the metal with some other substance present in traces in the glutathione. The free metal ions are clearly catalytically inactive in the oxidation of glutathione, and they are unable to form active complexes with the glutathione itself. (In most other iron-catalysed reactions it is generally supposed that the catalyst is an iron complex, and not free iron; for instance in the oxidation of cysteine the catalyst is a complex of iron with cysteine itself.) The oxidation then would depend on the co-operation of two factors—the metal, and the complex-forming substance. On this assumption, which was fully justified by later work, the slow rate of oxidation of the crystalline preparations is due to the very small amount of the complex-forming factor present in these preparations—even smaller than the amount of catalytic metals. This is therefore the limiting factor in this case, and the addition of further amounts of metals will have no effect on the velocity. With the impure glutathione preparations, on the other hand, where the extra substance is present in very much larger amount, the amount of metal will probably be small in comparison, and will become the limiting factor. In this case any extra metal added will be converted into the active complex, and the velocity will be increased.

In order to test this hypothesis we attempted to free glutathione from the catalytic impurity by recrystallisation. A sample of crystalline glutathione was recrystallised several times from water; and after this treatment it was found that the rate of oxygen uptake had fallen to a little over half that of the original preparation. (No oxidation of the glutathione had taken place during the recrystallisation, as the p_H was of course acid throughout, and it was carried out in evacuated vessels.) As metal-free water and vessels had been used in the recrystallisation it seemed possible at first that the diminished velocity was due to a diminution of the iron-content. The addition of iron to the solution had, however, no effect on the velocity, as shown in Fig. 3, so that the effect was clearly not due to the removal of iron. This experiment, while affording evidence of the removal of some factor (other than iron) upon which the reaction depends, showed that recrystallisation was not a satisfactory method for its removal.

Experiments were also carried out to determine whether the substance could be removed by adsorption methods. In preliminary experiments we found that, although substances such as cellulose or kieselguhr have no effect on the oxidation, the addition of kaolin to a solution of crystalline glutathione completely abolishes the oxygen uptake. This effect is, however, merely due to the removal of metals by adsorption on the kaolin, since the addition of iron to the filtrate restores the rate of oxygen uptake to precisely its original value. From the experiment shown in Fig. 4, it can be seen that the reaction is catalysed by iron, that the added iron is removed by kaolin, and that kaolin inactivates the glutathione merely by adsorbing metals and does not adsorb the other factor, since the velocity returns to its original value on restoring iron to the system.

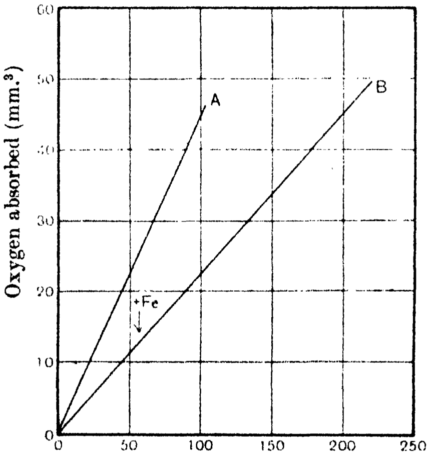

Fig. 3.

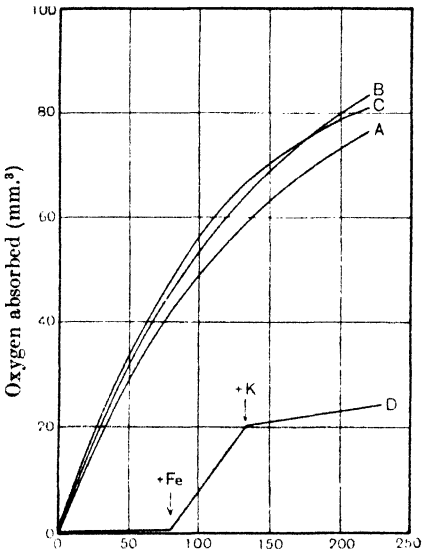

Fig. 4.

Fig. 3. Effect of recrystallisation. *A*, 3·5 mg. crystalline glutathione; *B*, 3·5 mg. recrystallised glutathione. In phosphate buffer at 20°. 0·0005 mg. iron added to *B* at arrow.

Fig. 4. Removal of iron by kaolin. *A*, 5 mg. crystalline glutathione; *B*, 5 mg. crystalline glutathione + 0·1 g. kieselguhr; *C*, 5 mg. crystalline glutathione + 0·1 g. filter paper; *D*, 5 mg. crystalline glutathione + 0·1 g. kaolin at 21°. 0·002 mg. Fe (as $FeSO_4$) added to *D* at first arrow. 0·1 g. more kaolin added at second arrow.

Treatment with kaolin therefore provides a very simple and convenient method for obtaining glutathione completely free from catalytic metals. All that is necessary is to add kaolin to the solution, shake gently for a short time, and filter. Kaolin does not, however, inactivate either cysteine or the earlier glutathione preparations (see Fig. 5), for reasons which will be discussed later.

With adsorbents of this nature we were unable to remove the auxiliary catalytic factor, but with certain adsorbents of a more biological nature we were completely successful in doing so. As, however, these experiments are closely connected with those on the behaviour of the tripeptide towards tissue systems, they will be dealt with in the following section.

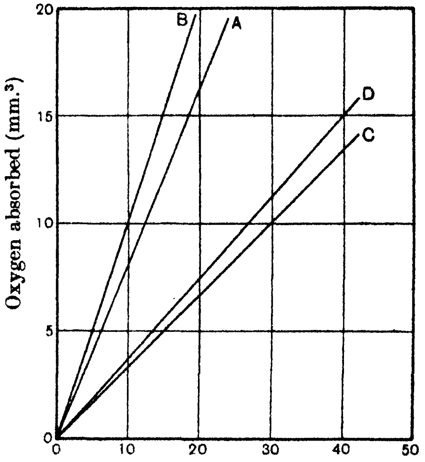

Fig. 5.

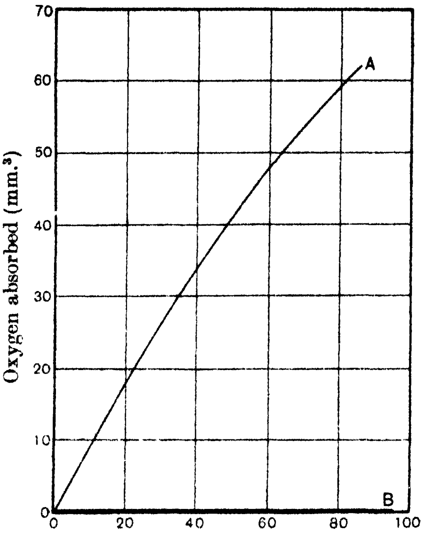

Fig. 6.

Fig. 5. Effect of kaolin on cysteine and on impure glutathione. *A*, 4 mg. old glutathione; *B*, 4 mg. old glutathione + 0·1 g. kaolin. In phosphate buffer at 40°. *C*, 5 mg. cysteine; *D*, 5 mg. cysteine + 0·1 g. kaolin. In phosphate buffer at 20°.

Fig. 6. Action of oxidised forms of old and new glutathione on thermostable tissue preparation. *A*, 4 mg. old oxidised glutathione + 0·2 g. muscle preparation; *B*, 4 mg. oxidised crystalline glutathione + 0·2 g. muscle preparation. In 3 cc. water at p_H 7·6 and 20°. In this and all other experiments the tissue was added to both flasks of the apparatus in order to eliminate the effect of any slight uptake by the tissue itself. The glutathione was of course added to the right-hand flask only.

Behaviour of glutathione towards tissue systems.

When the older preparations of glutathione, in either the reduced or the oxidised form, were added to thermostable muscle preparations at p_H 7·6 a large oxygen uptake resulted. In the case of the reduced form, the oxygen uptake was considerably greater than that given by the glutathione alone, owing to the simultaneous oxidation of protein. With the oxidised form, which of course gave no uptake in the absence of the tissue, the disulphide group was reduced by the —SH groups of the protein, and the oxygen uptake then followed as before.

Our first step in testing the behaviour of crystalline glutathione towards the thermostable system was to make certain that its oxidised form was capable of being reduced by the fixed —SH groups. For this purpose a solution of crystalline tripeptide in phosphate buffer was allowed to oxidise until it no longer gave a nitroprusside test. The resulting solution of oxidised glutathione was then added to a suspension of the muscle powder, allowed to stand for a short time, and then filtered. The filtrate gave a strong nitroprusside reaction, showing that pure oxidised glutathione, like the old, is readily reduced by the tissue powder.

In spite of this, however, the new glutathione shows a remarkable and complete difference in behaviour from the old: for on adding pure oxidised glutathione to the muscle powder not the slightest absorption of oxygen follows, although with the same muscle preparation the addition of the impure oxidised glutathione produces a large oxygen uptake. The results of a typical experiment are shown in Fig. 6.

It follows from this and similar experiments that pure glutathione is unable to bring about the oxidation of proteins. Moreover, since the tissue can reduce the glutathione it follows that reduced glutathione itself cannot oxidise in the presence of the muscle.

Yet more striking is the result when reduced crystalline glutathione is added to muscle powder. Not only is the tissue preparation itself not oxidised, but the oxidation of the glutathione is itself prevented by the presence of the tissue. The sulphydryl group of the tripeptide is in fact quickly rendered stable to oxygen by the muscle powder. During the first few minutes a small oxygen uptake is sometimes observed, as the stabilisation is not quite instantaneous, but this ceases almost immediately, and the system can then be kept for long periods of time without undergoing oxidation. The total oxygen uptake in any case is never greater than a very small fraction of that needed to oxidise the sulphydryl form to the disulphide. The experiment shown in Fig. 7 is typical of a large number of observations. For instance, out of 18 similar experiments with various preparations of tissue and of crystalline glutathione 14 showed rates of uptake (after the first few minutes) between 0 and 1 mm.^{3} per hour and the remaining 4 varied between 1 and 4 mm.^{3} per hour. The inactivation of the glutathione is therefore remarkably complete. The contrast between the behaviour of crystalline and impure glutathione is shown in Fig. 10.

After removal of the tissue by filtration the filtrate still shows no tendency to take up oxygen, as seen in Fig. 8. Nevertheless titration with iodine shows that it contains practically the whole of the reduced glutathione originally added; that is to say, only a small amount of the glutathione is adsorbed by the tissue.

200 mg. crystalline glutathione were dissolved in 60 cc. water and adjusted to p_H 7·6. 2 cc. of this were titrated with iodine (roughly $N/100$) using starch indicator. 2·57 cc. iodine were required. 2·5 g. thermostable muscle powder were then added to the solution, which was stirred for about an hour. After

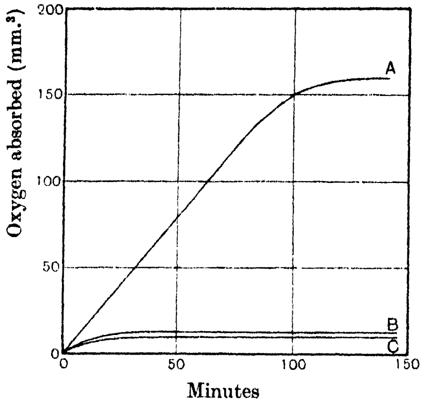

Fig. 7.

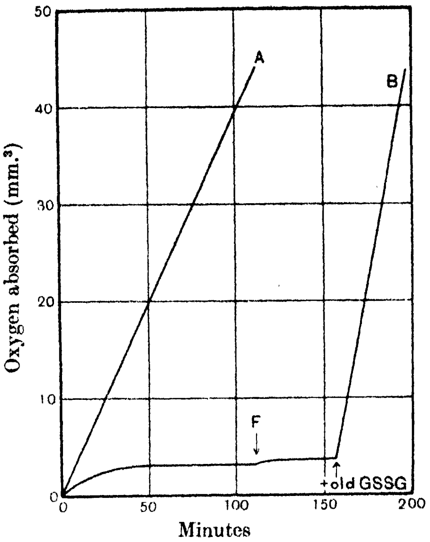

Fig. 8.

Fig. 7. Stabilisation of glutathione by thermostable tissue preparation. *A*, 8 mg. crystalline glutathione; *B* and *C*, 8 mg. crystalline glutathione + 0·2 g. muscle preparation. In water at p_H 7·6 and 41°.

Fig. 8. Effect of impure glutathione on stabilised glutathione. *A*, 5 mg. crystalline glutathione; *B*, 5 mg. crystalline glutathione + 0·2 g. muscle preparation. In buffer at p_H 7·3 and 16°. At the first arrow the tissue was removed by filtration, and at the second arrow 3 mg. impure oxidised glutathione were added to *B*.

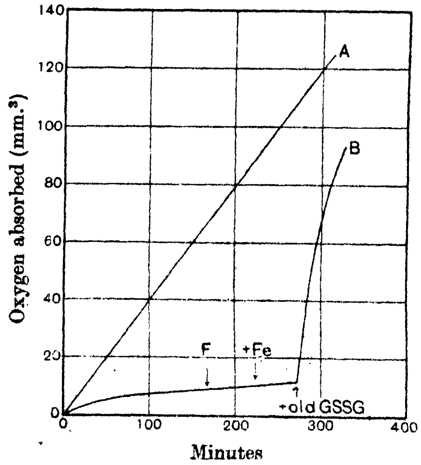

Fig. 9.

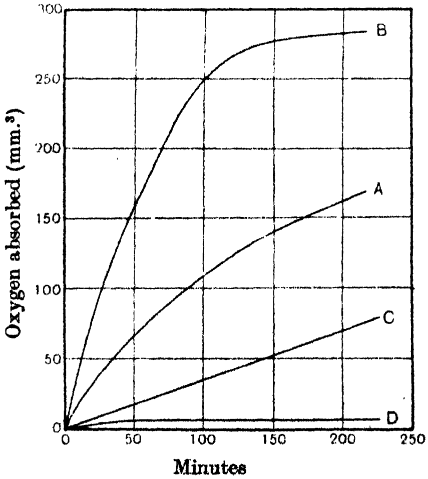

Fig. 10.

Fig. 9. Effect of iron on stabilised glutathione. *A*, 8 mg. crystalline glutathione; *B*, 8 mg. crystalline glutathione + 0·2 g. muscle preparation. In buffer at p_H 7·6 and 15°. At the first arrow the tissue was removed by filtration, at the second arrow 0·4 mg. Fe (as $FeSO_4$) was added, and at the third arrow 4 mg. impure oxidised glutathione.

Fig. 10. Comparative experiment with crystalline and impure glutathione in presence of tissue preparation. *A*, 9·6 mg. old (reduced) glutathione; *B*, 9·6 mg. old (reduced) glutathione + 0·2 g. muscle preparation; *C*, 5 mg. crystalline glutathione; *D*, 5 mg. crystalline glutathione + 0·2 g. muscle preparation. All in water at p_H 7·6 and 18°.

the tissue had been removed by centrifuging, 2 cc. of the solution required 2·33 cc. of the iodine. This tissue-treated solution, which of course gave a very strong nitroprusside reaction, was found in the Barcroft apparatus to give a quite negligible oxygen uptake.

Thus the addition of tissue powder to a solution of crystalline glutathione, followed after a short time by filtration, yields a solution of reduced glutathione which shows no tendency to undergo oxidation in air. We shall refer to glutathione which has been treated in this way as "stabilised glutathione."

We at first supposed that the stabilising effect of the muscle powder was similar to that of kaolin, and was due to the removal of metals by adsorption on the tissue. This is not the explanation, however, for whereas the addition of iron to the filtrate from the kaolin completely reactivated the glutathione, it had no effect upon the filtrate from the tissue, which remained quite stable to oxygen in the presence of even large amounts of iron or copper. Even such large amounts as 0·2 mg. of iron or copper have no effect. The effect of adding iron to stabilised glutathione is shown in Fig. 9, and precisely similar results were obtained with copper.

It is therefore quite clear that the tissue does not render the glutathione stable to oxygen by removing catalytic metals, but acts in some other way. Some evidence has already been given in the previous section that iron alone is not able to catalyse the autoxidation of glutathione; and this is convincingly shown by experiments of the type just described, for it is possible in this way to obtain strong solutions of reduced glutathione, containing large amounts of iron, which yet show no tendency to autoxidise. It is clear that iron and copper salts are themselves catalytically inactive in the reaction and do not form catalytic complexes with pure glutathione. As we suggested in the previous section, it appears that another factor must be present in addition to the iron in order that complexes capable of catalysing the oxidation of glutathione may be formed. If this is so the most probable explanation of the stabilising action of the muscle powder is that it removes the additional factor from the solution by adsorption. Glutathione can thus be rendered incapable of autoxidation in two ways: by the removal of metals by treatment with kaolin, or by removal of the other factor by treatment with muscle powder. In the first case the addition of iron to the filtrate reactivates it, since the second factor is not removed; in the second case this is lacking and the filtrate is therefore not reactivated by iron. It should, however, be reactivated by the addition of impure glutathione, which appears to contain the factor in considerable amount, as stated in the last section. The marked effect of adding glutathione from an earlier preparation to the stabilised glutathione is shown in Figs. 8 and 9.

The stabilised glutathione has lost not only its power to react with oxygen, but also its power of reducing methylene blue. (The reduction of methylene blue by cysteine was shown by Toda [1926] and by Harrison [1927] to be catalysed by metals, like the autoxidation.) The following experiment shows the effect of previous treatment with tissue upon the velocity of reduction.

A solution of crystalline glutathione in phosphate buffer, adjusted to p_H 7·6 and containing 4 mg. glutathione per cc., was prepared. This was divided into two portions: one portion was stabilised by treatment with muscle powder, which was then removed after about $\frac{3}{4}$ hour by filtration; the other portion was meanwhile kept in an evacuated Thunberg tube to prevent oxidation. Equal amounts of the two solutions, together with phosphate buffer, were then added to two Thunberg tubes so that each tube contained 3 mg. glutathione in 2 cc. liquid, then 0·3 cc. of 1/5000 methylene blue was added to each, and the reduction-times determined at room temperature in the usual way. An old preparation of glutathione under these conditions gave a reduction-time of about 40 minutes; the crystalline glutathione reduced in about 3 hours, while the stabilised glutathione did not reduce in 15 hours.

The behaviour of the —SH group of stabilised glutathione, which on our view is simply pure glutathione, resembles that of the fixed —SH groups of the thermostable preparations in being unable to react with either oxygen or methylene blue, even in presence of iron, and in becoming readily oxidised by both in the presence of impure glutathione.

Fig. 10 shows a comparative experiment with old and new glutathione preparations on the same sample of tissue. It confirms the results of Hopkins's experiments with the earlier preparations, and illustrates the marked difference in the action of the crystalline and the impure preparations. The reason for the failure of the tissue to stabilise the older preparations seems to be simply that they contain far too much of the auxiliary catalytic factor for the whole of it to be adsorbed by the tissue, so that the tissue becomes saturated with it before more than a fraction is removed from solution. Evidence for this will be given later.

Both cysteine and thioglycollic acid behave like the non-crystalline glutathione preparations, as Fig. 11 shows. This is to be expected, since the tissue does not remove the iron from the solution, and these substances are themselves capable of forming active complexes with iron. It is only those sulphydryl compounds which are unable to form such complexes, and require the presence of another factor to activate the iron, which become stabilised.

Since it appears that cysteine can form such complexes and glutathione cannot, we thought it probable that other peptides of cysteine might also be incapable of forming active metal complexes, and either be stable to oxygen or become stabilised by tissue. On testing glycylcysteine[1] we found that it oxidised spontaneously at a slow rate, which was practically unaffected by the addition of iron, and that the addition of tissue powder had exactly the same stabilising effect as with crystalline glutathione. This experiment is shown in Fig. 12. This result seems to indicate that cysteine peptides generally may resemble glutathione in being unable to form complexes with metals which are able to catalyse their oxidation.

Chloroacetylcystine was also tested, and found to behave like pure oxidised glutathione, that is to say, when it was added to tissue no oxygen uptake could be detected, although a nitroprusside test on the filtrate showed that reduction of the disulphide group by the tissue had taken place. The chloroacetylcysteine formed was therefore stable to oxygen in presence of tissue.

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[1] We are much indebted to Mr N. W. Pirie for giving us a sample of this peptide.

The action of haematin on stabilised and crystalline glutathione is very similar, for in both cases small amounts have no accelerating effect but large amounts (*e.g.* 0·5 mg.) definitely produce an acceleration. Fig. 13 shows the effect of adding a large amount of haematin to glutathione in the presence and absence of tissue preparation. While the inactivation is partially prevented by the presence of the haematin, the inhibitory effect of the tissue is still very marked.

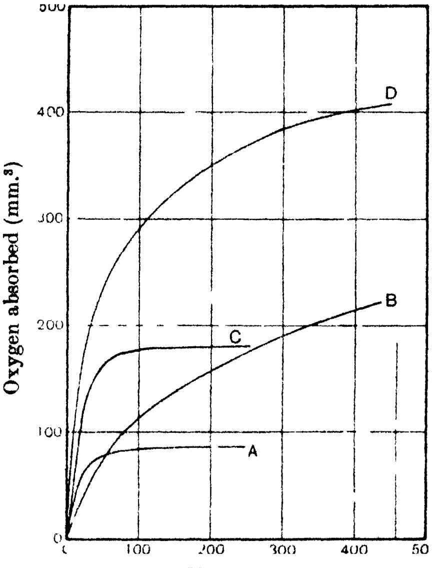

Fig. 11.

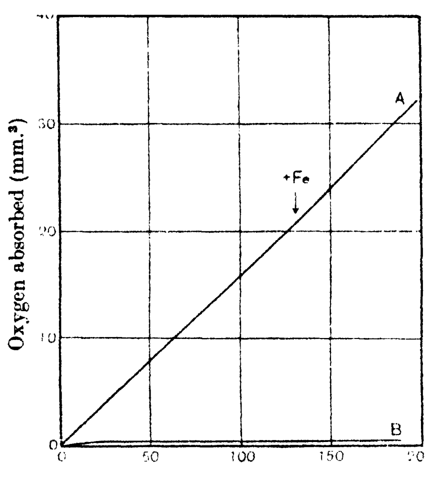

Fig. 12.

Fig. 11. Action of cysteine and thioglycollic acid on tissue preparation. *A*, 2 mg. cysteine + 0·001 mg. Fe; *B*, 2 mg. cysteine + 0·001 mg. Fe + 0·2 g. muscle preparation; *C*, 3 mg. thioglycollic acid; *D*, 3 mg. thioglycollic acid + 0·2 g. muscle preparation. All in water at p_H 7·6 and 16°. The oxygen-equivalents of the cysteine and thioglycollic acid respectively are indicated by the vertical lines on the right of the figure.

Fig. 12. Stabilisation of glycylcysteine. *A*, 5 mg. glycylcysteine; *B*, 5 mg. glycylcysteine + 0·2 g. muscle preparation. In phosphate buffer at p_H 7·6. At the arrow 0·001 mg. Fe was added.

In further experiments on the stabilisation of glutathione by tissue preparations it was found that the p_H of the solution had a considerable influence, for while at p_H 7·6 the stabilisation was usually complete no stabilisation at all could be detected at p_H 9·0. The presence of the tissue had in fact no effect on the velocity of oxidation in alkaline solution. A solution of glutathione which had been stabilised at p_H 7·6 did not become reactivated by making it alkaline, so that it appears that the tissue does not adsorb the catalytic impurity in alkaline solutions. The significance of this result will appear later.

The thermostable tissue preparation consists mainly of insoluble protein material, and we therefore investigated the action of other proteins upon the rate of oxidation of aqueous glutathione solutions, in order to see whether they showed the same stabilising effect as the tissue powder. It was found that gelatin, caseinogen and egg-albumin did not affect the rate of oxygen uptake, but that coagulated egg-albumin slowed it considerably. At first it appeared possible that the presence of a sulphydryl group in the protein molecule might be essential for it to exert a stabilising action, for the tissue preparations and coagulated egg-albumin possess sulphydryl groups, whereas gelatin, caseinogen and native egg-albumin do not. This was, however, shown not to be the case by the fact that denatured globin[1], which shows no —SH reaction, stabilised the glutathione almost completely. It appears that the heterogeneity of the system is the determining factor. Some of these experiments are shown in Fig. 14.

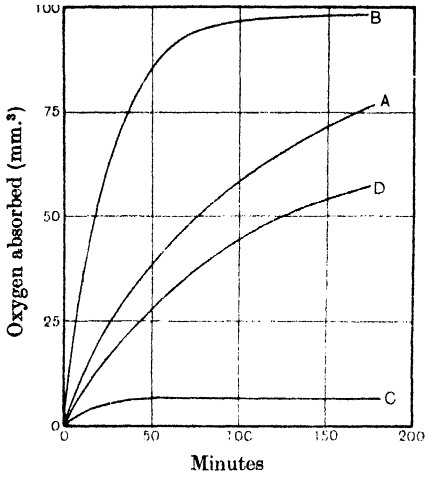

Fig. 13.

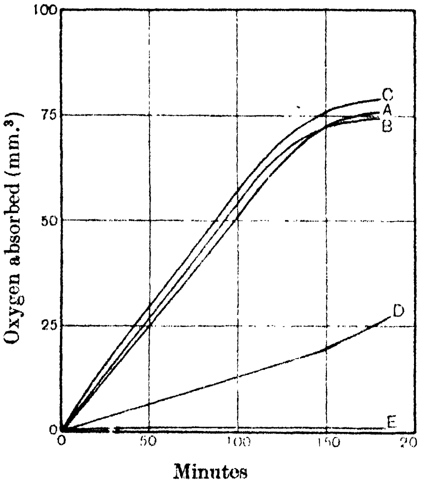

Fig. 14.

Fig. 13. Effect of larger amounts of haematin. *A*, 5 mg. crystalline glutathione; *B*, 5 mg. crystalline glutathione + 0·5 mg. haematin; *C*, 5 mg. crystalline glutathione + 0·2 g. muscle preparation; *D*, 5 mg. crystalline glutathione + 0·5 mg. haematin + 0·2 g. muscle preparation. All in buffer at p_H 7·6 and 20°. The tissue used in this experiment had been freed from haematin by extraction with pyridine, followed by ether.

Fig. 14. Effect of proteins on autoxidation of glutathione. *A*, 5 mg. crystalline glutathione; *B*, 5 mg. crystalline glutathione + 0·04 g. gelatin; *C*, 5 mg. crystalline glutathione + 0·2 g. egg-albumin; *D*, 5 mg. crystalline glutathione + 0·2 g. denatured egg-albumin; *E*, 5 mg. crystalline glutathione + 0·07 g. denatured globin. All in phosphate buffer at p_H 7·6 and 16°.

From the biological point of view it was obviously important to determine whether fresh unheated tissues could also stabilise glutathione, or whether this property was restricted to tissue preparations which had been heated. It was possible that the power to remove the catalytic factor by adsorption had been conferred upon the thermostable preparations by the heat-coagulation of their proteins. In order to test this, fresh rat muscle was minced, well washed with tap water, and added to a solution of crystalline glutathione. The results of one experiment are shown in Fig. 15. In another experiment with a different rat a larger inhibition of oxygen uptake was obtained. The fact that contact with fresh tissues tends to render glutathione stable to oxygen cannot but be of considerable importance in connection with the mode of action of glutathione *in vivo.*

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[1] We are indebted to Mr R. Hill for samples of globin prepared from horse and sheep blood.

Oxidation of unsaturated fatty acids.

So far we have been dealing with the experiments on the oxidation of protein, in which the oxidation of the sulphydryl group of glutathione is involved. Hopkins also found that glutathione could catalyse the oxidation of unsaturated fats and fatty acids, particularly in acid solution. In this catalysis the oxidation of the —SH group is not involved, and there was therefore no reason to anticipate that the phenomena connected with stabilisation should affect this action. In fact the behaviour of the crystalline glutathione towards fats appears to be exactly the same as the non-crystalline. In Fig. 16 is shown a comparative experiment on linoleic acid at p_H 3·5 with old and new glutathione. It will be seen that the induced oxygen uptake is the same in both cases.

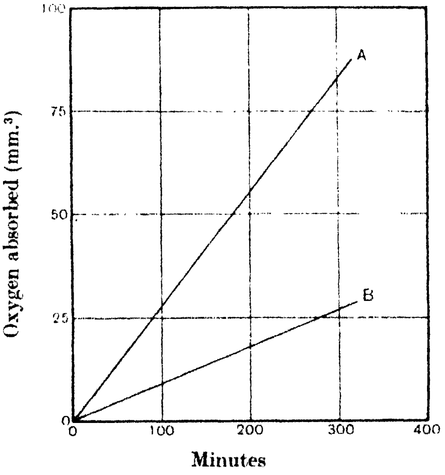

Fig. 15.

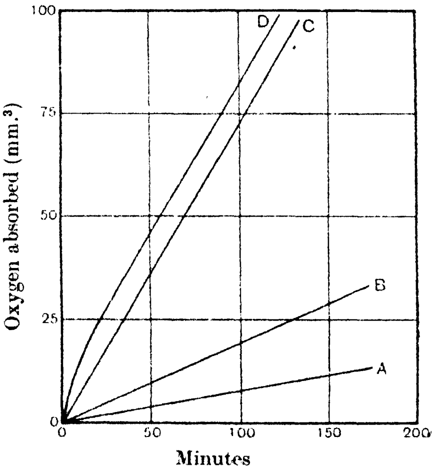

Fig. 16.

Fig. 15. Inhibition of autoxidation of glutathione by washed rat muscle. *A*, 5 mg. crystalline glutathione + 0·001 mg. Fe; *B*, 5 mg. crystalline glutathione + 0·001 mg. Fe + 1 g. washed rat muscle. In phosphate buffer at p_H 7·6 and 16°. The muscle and iron were added to both flasks of the apparatus, so as to compensate for any residual respiration.

Fig. 16. Oxidation of linoleic acid by old and new glutathione preparations. *A*, 8 mg. crystalline glutathione; *B*, 8 mg. impure glutathione; *C*, 10 mg. linoleic acid + 8 mg. crystalline glutathione; *D*, 10 mg. linoleic acid + 8 mg. impure glutathione. All in water at p_H 3·5 and 39°. The linoleic acid was present as a fine emulsion, and it was added to both flasks of each apparatus *C* and *D*, in order to compensate for its spontaneous autoxidation.

Several samples of linseed oil were also tested at p_H 7·6, but with the samples available we obtained no oxidation either with pure or impure glutathione or with cysteine, thus confirming the results of Allott [1927].

We have not observed any differences in behaviour between the old and new glutathione preparations as far as the oxidation of fats is concerned.

Nature of the catalytic impurity. Reactivation of stabilised glutathione.

Since the catalytic substance is present in crystalline glutathione only in extremely small amounts (quite insufficient to affect the analytical figures) it is not practicable to isolate it and identify it directly. Evidence as to its nature must therefore mainly rest upon the comparison of its behaviour with that of known substances. While we cannot claim to have definitely identified the substance, we have obtained evidence which gives distinct indications of its nature; and it appears very probable that it is either free cysteine or some very closely related substance. Its properties correspond almost exactly with those of free cysteine; nevertheless certain quantitative experiments seem to show that its activity is somewhat greater than that of cysteine itself.

Cysteine is known to be capable of forming active complexes with iron and copper, it is known to be present in considerable amounts in the older glutathione preparations, it is the most likely impurity to occur in the new glutathione preparations (since it is precipitated by the Hopkins copper method), and it would probably also occur in traces in preparations of other cysteine peptides.

On this hypothesis then the catalyst which brings about the oxidation of glutathione is a cysteine-iron (or copper) complex. According to the theory of cysteine autoxidation recently put forward by Michaelis [1929] the oxidation of cysteine is due to the catalytic action of a cysteine-iron complex rather than of iron itself. In that case both glutathione and cysteine would be oxidised by the same catalyst. This would explain a fact which has hitherto been somewhat difficult to account for; namely, that the p_H-oxidation-velocity curves for cysteine and (impure) glutathione (in presence of a trace of copper) have the same form, with a marked optimum at p_H 7·5, although the mode of ionisation of the two substances is different. Other sulphydryl compounds, such as thioglycollic acid, show quite different curves, with no optimum in this region. This would seem to indicate that the catalyst is the same in both cases.

We were first led to think that the extra catalytic factor might be cysteine by the following circumstance. In preparing glutathione by Hopkins's [1929] method, if the final solution, obtained by the decomposition of the crystalline copper compound by means of H_2S, is quickly concentrated *in vacuo* and allowed to crystallise after adding alcohol in the usual way, the whole of the glutathione is obtained as a non-deliquescent crystalline preparation, with the properties described in this paper. If, however, the solution is allowed to stand for a few hours, or the evaporation carried out slowly, the preparation obtained is only partially crystalline and resembles the older preparations in being deliquescent. Slight hydrolysis of the tripeptide appears to have taken place with the production of a small amount of free cysteine; as shown by the fact that the Sullivan [1926] test, which is negligibly weak with crystalline glutathione, became definitely positive with this preparation. The products of hydrolysis apparently interfere with the crystallisation of the tripeptide. This preparation, which we shall refer to as "deliquescent glutathione," and which we may repeat is obtained from the crystalline copper compound of the tripeptide, behaves with tissue powder quite differently from the crystalline preparations, but in very much the same way as the older preparations, as shown in Fig. 17. It appeared therefore that glutathione containing a sufficient amount of free cysteine to give a positive Sullivan reaction, as in the case of the older and the deliquescent preparations, was not stabilised by the tissue; whereas preparations which gave a negative Sullivan reaction were stabilised. That is to say, where the glutathione contained more than a minute trace of cysteine the active factor was present in too large an amount to be removed by adsorption on the tissue.

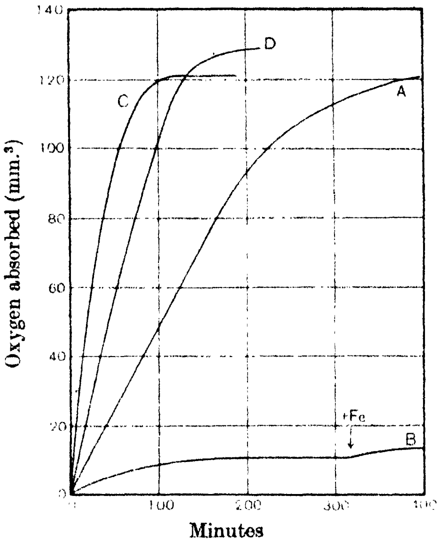

Fig. 17.

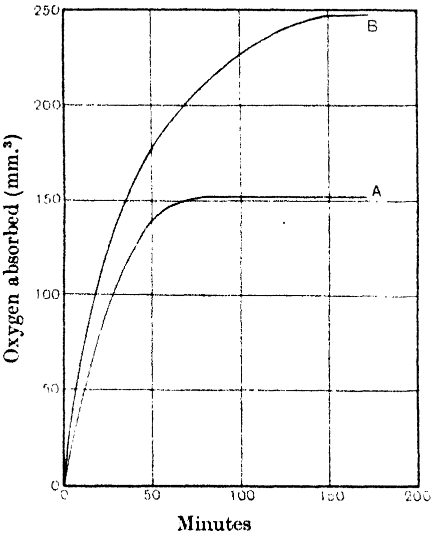

Fig. 18.

Fig. 17. Action of tissue preparation on crystalline and "deliquescent" glutathione. *A*, 7 mg. crystalline glutathione; *B*, 7 mg. crystalline glutathione + 0·2 g. muscle preparation; *C*, 8 mg. "deliquescent" glutathione; *D*, 8 mg. "deliquescent" glutathione + 0·2 g. muscle preparation. In phosphate buffer at p_H 7·6 and 20°. 0·01 mg. Fe (as $FeSO_4$) added to *B* at arrow.

Fig. 18. Action of "reactivated" glutathione on thermostable tissue preparation. *A*, 10 mg. "reactivated" glutathione: *B*, 10 mg. "reactivated" glutathione + 0·2 g. muscle preparation. In water at p_H 7·5 and 40°.

This experiment suggested that crystalline glutathione which had been allowed to stand in aqueous solution for some time might behave similarly. A solution containing 100 mg. crystalline glutathione in 10 cc. water was accordingly allowed to stand (unneutralised) for 12 hours at 40°, after which it was adjusted to p_H 7·5. It was then found that the solution gave a definitely positive Sullivan reaction, indicating that a small amount of hydrolysis had taken place. The oxygen uptake of this solution was accelerated by adding a trace of iron, like that of the impure glutathione, and it also behaved like the latter with tissue: that is to say its oxidation was not inhibited by the tissue, and it was able to catalyse the oxidation of the protein, as shown in Fig. 18. This experiment has been repeated with other crystalline preparations with similar results.

These experiments show clearly that the catalytic factor can be formed from the pure tripeptide itself, and that its appearance is accompanied by the liberation of a small amount of free cysteine.

We attempted to make use of the delicate and specific Sullivan test for free cysteine for the purpose of estimating colorimetrically the amounts present in the different preparations of glutathione, but we found that in presence of glutathione the development of the colour is markedly inhibited. For instance 1 mg. of cysteine gave a strong Sullivan reaction, but 1 mg. of cysteine plus 9 mg. of glutathione gave only a weak colour. For this reason it is difficult to use the Sullivan method for the estimation of small amounts of cysteine in glutathione preparations. It can, however, be said that there are indications that the strength of the reaction runs roughly parallel with the amount of catalytic factor present. All the older glutathione preparations tested gave a strong (though rather variable) test, the deliquescent preparations and such solutions as that used in Fig. 18 gave positive, though weaker, tests, while crystalline glutathione gave only a negligible colour. Attempts to obtain quantitative results by using a series of standards containing varying amounts of cysteine and glutathione were not very successful, but appeared to show that between 1 and 5 % of the total sulphydryl in the solutions used in Fig. 18 was present as free cysteine.

We may mention here that the addition of ferric chloride to the impure glutathione produces the transient blue colour characteristic of cysteine, while with crystalline glutathione or with glycylcysteine no blue colour is produced, and only a pale olive tint remains.

In order that the hypothesis that the catalytic substance is cysteine may be tenable, the following points must be verified. (1) A mixture of crystalline glutathione with a small proportion of cysteine must behave like the non-crystalline glutathione preparations. (2) The thermostable muscle powder must be capable of removing small amounts of cysteine from solution by adsorption. (3) The addition of cysteine to stabilised glutathione must reactivate it.

All these points have been verified by experiment.

Fig. 19 shows that the addition of cysteine to crystalline glutathione prevents the latter from being inactivated by the tissue; and the large effect of adding small quantities of cysteine to reduced or oxidised glutathione is illustrated in Fig. 20.

The presence of phosphate tends to prevent the oxidation of protein [see Hopkins, 1925], but Fig. 21, which gives an experiment carried out in the absence of phosphate, shows that extended oxygen uptakes with oxidation of protein can be obtained on adding a trace of cysteine to the crystalline glutathione.

With regard to the second point, when very small amounts (less than 0·1 mg.) of cysteine are added to 0·2 g. of muscle powder at p_H 7·6 no oxygen uptake is produced. On filtering off the tissue, the liquid is found to give no nitroprusside reaction, even after adding cyanide to reduce any cysteine which may have been formed. The cystine has in fact been completely removed from the solution by adsorption on the tissue. This does not occur, however, in alkaline solutions: at p_H 9 the tissue does not adsorb the cysteine. Thus

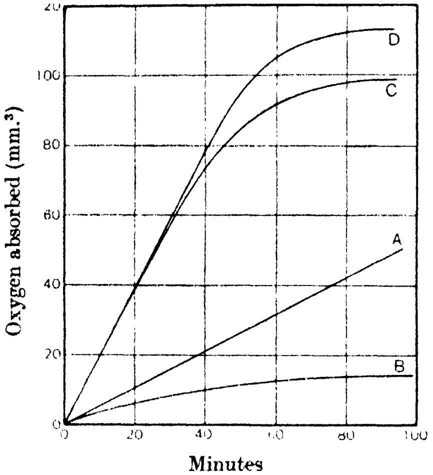

Fig. 19. Prevention of stabilisation by presence of cysteine. *A*, 5 mg. crystalline glutathione; *B*, 5 mg. crystalline glutathione + 0·2 g. muscle preparation; *C*, 5 mg. crystalline glutathione + 1 mg. cysteine; *D*, 5 mg. crystalline glutathione + 0·2 g. muscle preparation + 1 mg. cysteine. In phosphate buffer at p_H 7·6 and 20°. The cysteine and muscle were added to both flasks of the apparatus. The uptake of the cysteine itself is therefore eliminated from the curves.

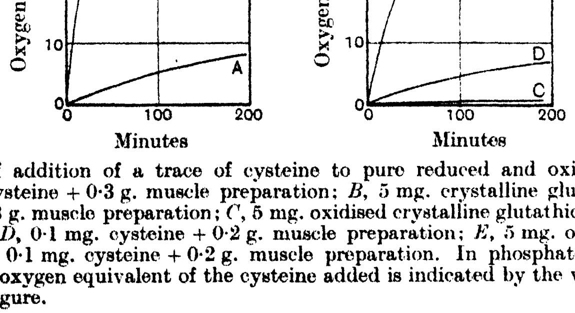

Fig. 20. Effect of addition of a trace of cysteine to pure reduced and oxidised glutathione. *A*, 0·1 mg. cysteine + 0·3 g. muscle preparation; *B*, 5 mg. crystalline glutathione + 0·1 mg. cysteine + 0·3 g. muscle preparation; *C*, 5 mg. oxidised crystalline glutathione + 0·2 g. muscle preparation; *D*, 0·1 mg. cysteine + 0·2 g. muscle preparation; *E*, 5 mg. oxidised crystalline glutathione + 0·1 mg. cysteine + 0·2 g. muscle preparation. In phosphate buffer at p_H 7·6 and 40°. The oxygen equivalent of the cysteine added is indicated by the vertical line on the right of the figure.

under conditions where the muscle powder adsorbs cysteine it is capable of stabilising glutathione, and under other conditions, where it cannot stabilise glutathione, it does not adsorb cysteine.

Moreover the muscle powder may be rendered incapable of adsorbing cysteine by previously exposing it to excess of cysteine so as to saturate it, and then removing the free cysteine by very thorough washing with water. After this treatment the tissue was incapable of stabilising glutathione.

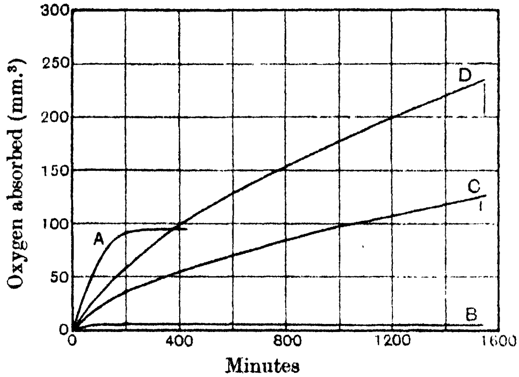

Fig. 21. Oxidation of protein by crystalline glutathione in presence of small amounts of cysteine. *A*, 5 mg. crystalline glutathione; *B*, 5 mg. crystalline glutathione + 0·2 g. muscle preparation; *C*, 5 mg. crystalline glutathione + 0·2 g. muscle preparation + 0·25 mg. cysteine; *D*, 5 mg. crystalline glutathione + 0·2 g. muscle preparation + 1 mg. cysteine. In water at p_H 7·6 and 18°. The cysteine was added to both flasks in *C* and *D*. The vertical lines indicate the oxygen equivalent of the amounts of cysteine added.

Another point of interest is that in the presence of excess of glutathione the tissue seems to have much less power of adsorbing cysteine. This is possibly because it can adsorb both compounds so that in presence of an excess of glutathione the surface would be mainly saturated with this and little cysteine would be adsorbed. Fig. 22 illustrates this point. Neither the cysteine nor the glutathione separately gave any oxygen uptake in presence of the tissue: the cysteine because it was present in sufficiently small quantity to be completely adsorbed by the tissue, the glutathione because it became stabilised by the tissue in the usual way. When both were present together, however, a considerable oxygen uptake was produced.

With regard to the third point, the addition of a trace of cysteine to stabilised glutathione (after the tissue has been filtered off) restores the power to take up oxygen. The effect of adding different amounts of cysteine is shown in Fig. 23.

All the evidence given hitherto in this section seems to indicate that the impurity necessary for the oxidation of glutathione is cysteine. The fact that kaolin removes the catalytic iron from glutathione preparations but not from cysteine might be thought to be evidence to the contrary, since this view supposes that the active iron is present in both cases as a complex with cysteine.

But the difference is easily understood if we assume that the complex is partly dissociated into free iron and cysteine, and that the kaolin only adsorbs free iron. For in accordance with the principle of mass action the amount of free iron will decrease as the cysteine concentration increases; so that in the glutathione solutions, where the cysteine is present only in traces, the kaolin may be able to remove practically the whole of the iron, while in the cysteine solutions the concentration of free iron will be negligible, and therefore very little will be adsorbed.

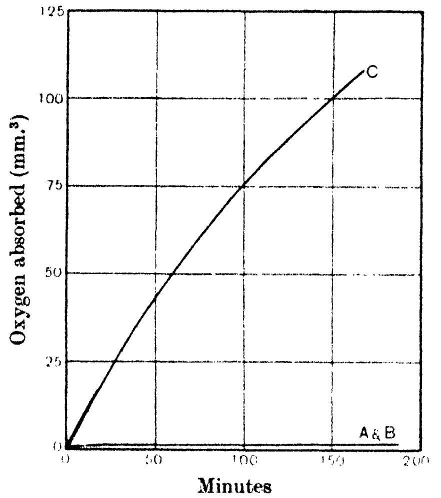

Fig. 22.

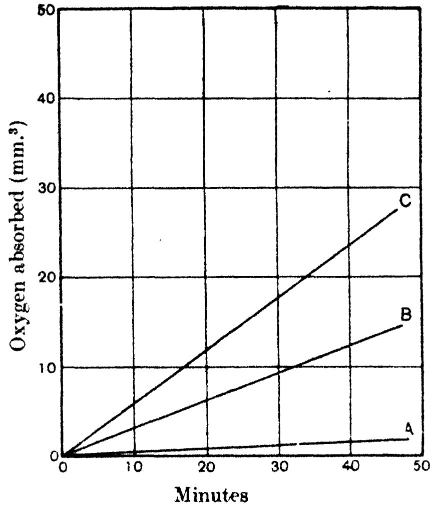

Fig. 23.

Fig. 22. Effect of very small amounts of cysteine. *A*, 5 mg. crystalline glutathione + 0·2 g. muscle preparation; *B*, 0·05 mg. cysteine + 0·2 g. muscle preparation; *C*, 5 mg. crystalline glutathione + 0·2 g. muscle preparation + 0·05 mg. cysteine. In water at p_H 7·6 and 40°. The oxygen-equivalent of the cysteine added was about 2 $mm.^3$ It was found that in the absence of the glutathione the tissue completely adsorbed the cysteine.

Fig. 23. Effect of addition of cysteine to stabilised glutathione. A solution of stabilised glutathione was prepared by treatment with thermostable preparation, followed by filtration. Various amounts of cysteine were then added to the filtrate as follows: *A*, 2 mg. stabilised glutathione + 0·001 mg. Fe; *B*, 2 mg. stabilised glutathione + 0·001 mg. Fe + 0·1 mg. cysteine; *C*, 2 mg. stabilised glutathione + 0·001 mg. Fe + 0·2 mg. cysteine. In phosphate buffer at p_H 7·6 and 20°. The cysteine and iron were added to both flasks of the apparatus.

Quantitative experiments seem to show that while cysteine (together with iron) can act as catalyst in the oxidation of glutathione, its activity is somewhat less than that of the active substance present in solutions of the crystalline, "deliquescent" and "reactivated" glutathiones. The results given in Fig. 23 show that the presence of 5 % of free cysteine in the glutathione would account for an oxygen uptake of about 50 $mm.^3$ per hour for 5 mg. of glutathione. Now the rate of oxidation of crystalline glutathione under similar conditions is about 30 $mm.^3$ per hour per 5 mg. If this oxidation were due to the presence of cysteine, crystalline glutathione must contain 3 % of free cysteine. This, however, does not seem possible. The limits of error of the analytical figures would only permit of the presence of 0·5 % of free cysteine at most (assuming, however, that cysteine is the only impurity present), and an even smaller amount than 0·5 % could have been detected by the Sullivan reaction in spite of the inhibitory effect of the glutathione. Similar reasoning shows that the "deliquescent" glutathione used in the experiment of Fig. 17 would have to contain about 8 % of free cysteine, whereas the Sullivan reaction indicated that not more than about 1 % was present. Added cysteine therefore hardly seems to be sufficiently active.

Nevertheless in view of the somewhat complicated nature of the reactions involved we do not feel justified in claiming that these considerations are conclusive.

DISCUSSION.

We consider that the experiments given in this paper show that pure glutathione is not appreciably autoxidisable, even in presence of iron.

There are four possible interpretations of the effect of the muscle powder in preventing the oxidation of glutathione. (1) The tissue may remove the necessary iron by adsorption. (2) Some substance which inhibits the catalytic action of the iron may pass into the solution from the tissue. (3) The glutathione may be converted into a non-autoxidisable isomeric modification, the tissue acting as a catalyst in the process. (4) The tissue may remove from the solution some impurity other than iron, which co-operates with the iron in catalysing the oxidation.

The first possibility is disposed of by the fact that the addition of iron after filtering off the tissue powder does not render glutathione autoxidisable.

The second is made improbable in view of the very thorough extraction undergone by the tissue preparation. The tissue is first very thoroughly extracted with water, then with boiling water, then with absolute alcohol for several hours in a Soxhlet apparatus, and finally with ether. In some cases it was also extracted with pyridine and again with ether. After such treatment it is difficult to believe that any soluble material is left.

This interpretation seems also to be excluded by the form of the curves. The stabilisation is not instantaneous, but takes a certain time to become established. The initial velocity of oxidation in presence of tissue is not much below that in its absence, but it quickly falls off to a negligible value. On the second interpretation this period would correspond to the time taken for the inhibitor to diffuse out of the tissue. If therefore the tissue is allowed to remain in the buffer solution for say 2 hours before adding the glutathione, the inhibitor should have had time to diffuse into the solution, and the stabilisation should now be instantaneous. Actually, however, this procedure has no effect on the length of time taken to attain complete stabilisation.

The hypothesis is finally disposed of by the following consideration. In a series of experiments with different amounts of tissue it was found that 0·08 g. of the tissue was required in order to stabilise 5 mg. of glutathione. Smaller amounts of tissue produced only a partial inhibition. On the hypothesis that the tissue was supplying an inhibitor this would mean that that amount of tissue contained just enough of the inhibitor to inactivate the metals present. The amount of tissue used in most of our experiments, 0·2 g., would therefore only be able to inactivate about twice as much iron as was originally present in the solution. Yet after removing the tissue by filtration, many (*e.g.* 1000) times this amount of iron can be added without re-establishing any uptake of oxygen. The second interpretation may therefore be neglected.

In order to explain the stabilisation of glutathione by its conversion into an isomeric modification it would seem to be necessary to postulate some change involving the sulphydryl group, so that stabilised glutathione would not contain a free sulphydryl group. Any change not involving the sulphydryl group would by itself hardly be expected to prevent the autoxidation. Yet stabilised glutathione does not appear to have undergone any change in its molecule. It titrates normally with iodine, it is precipitated normally by the copper method, and it gives with nitroprusside a sulphydryl reaction of undiminished intensity. It is true that in performing the nitroprusside test the solution is made alkaline with ammonia, which might produce a tautomeric change; but we found that this treatment did not reactivate stabilised glutathione in the least. Moreover on this view the tissue powder would be acting as a catalyst for the change, and the rate of stabilisation should vary with the amount of tissue present. Actually the time taken to reach stabilisation is independent of the amount of tissue within wide limits. And finally this hypothesis provides no explanation of the reactivation of the stabilised glutathione by the addition of a little impure glutathione or cysteine.

According to the remaining hypothesis the tissue removes some substance which, in co-operation with iron, is responsible for the catalysis. It has been clearly shown that iron alone cannot catalyse the oxidation of the cysteine peptides, neither can it form active complexes with them. Nevertheless that the oxidation depends upon the action of iron is shown by the experiments with kaolin. We are therefore compelled to assume that the active catalyst is a complex of iron with some other impurity, in the absence of which glutathione would be stable to oxygen, even in presence of iron. Several lines of evidence have been shown in this paper to converge in support of this view, and it appears to provide an adequate explanation of the facts.

The fact that iron and copper can form active complexes with cysteine but not with the cysteine peptides is of importance in connection with theories of oxidation-catalysis by iron. It appears that the amino-group of cysteine must be left free in order that active complexes with iron may be formed.

With regard to the nature of the impurity in crystalline glutathione which forms active complexes with iron, we can only say that its behaviour is similar to that of free cysteine. The identity of the impurity in these preparations is perhaps not a matter of very great consequence. In any case cysteine is capable of acting in such a way, and there can be little doubt that in the work done on the autoxidation of the older glutathione preparations (as for instance that of

Dixon and Tunnicliffe [1923], Harrison [1924], Dixon [1928]) the effects were due to the presence of cysteine, as also in the case of the oxidations of proteins studied by Hopkins [1925].

We do not picture the cysteine impurity as acting by the alternate oxidation and reduction of its sulphur group, but merely as converting the iron into a catalytically active complex. It is the sulphur group of glutathione which undergoes alternate oxidation and reduction in the presence of tissue, the ferrocysteine catalysing the reaction between glutathione and oxygen.

The question now arises as to how glutathione is oxidised in the tissues. The following possibilities must be considered. (1) Autoxidation catalysed by ferrocysteine or similar complexes. (2) Autoxidation catalysed by haematin. (3) Oxidation by means of the cytochrome system. (4) Oxidation by hydrogen peroxide, either directly, or indirectly as suggested by Szent-Györgyi [1928].

The first method seems to be the most probable. It does not seem to be definitely known at present whether the tissues contain free cysteine in appreciable quantities or not, but it seems quite probable that sufficient amounts would be present to account for the oxidation.

We have shown that small amounts of haematin do not affect the autoxidation, and it seems questionable whether sufficient haematin is present in the cells to catalyse the reaction.

Keilin [1930] has shown that cysteine is very rapidly oxidised by the cytochrome—indophenol oxidase system, with uptake of oxygen, and it might be expected that glutathione would be oxidised by the same system. Dr Keilin has kindly allowed us to state, however, that he has found that crystalline glutathione is not oxidised by this system, and that it reduces cytochrome only with extreme slowness (cf. our results on haematin given previously).

Further work is at present being carried out on the possibility of oxidation by hydrogen peroxide.

With the data at present available no final statement can be made with regard to the mode of oxidation of glutathione in the tissues.

SUMMARY.

1. Previous work on oxidation-catalysis by glutathione, in which impure preparations were used, has been repeated with crystalline glutathione. Some significant differences in behaviour have been found.

2. Solutions of crystalline glutathione absorb oxygen at the comparatively slow rate of about 30 mm.^{3} per hour per 5 mg. glutathione (at p_H 7·6 and 20°).

3. As in the case of cysteine, this oxidation is due to the presence of traces of catalytic metals. These can be removed by adsorption on kaolin. The resulting solutions do not absorb oxygen, but are reactivated by the addition of a trace of iron, which brings back the autoxidation to its original rate. Cysteine is not inactivated by kaolin.

4. The autoxidation of solutions of crystalline glutathione, unlike that of cysteine, is not accelerated by the addition of iron.

5. Evidence has been obtained that the autoxidation of glutathione depends on the co-operation of two factors, present in traces as impurities in the glutathione preparations; namely, iron (or copper), and some substance able to form catalytically active complexes with metals. With crystalline glutathione the rate of oxidation is limited by the amount of the second factor present, and not by the iron.

6. Crystalline glutathione, unlike the earlier preparations, produces no oxygen uptake when added to "thermostable tissue preparations." When the disulphide form is added it becomes reduced by the tissue, but no oxygen uptake results. When the sulphydryl form is added not only does it fail to oxidise the protein, but its own autoxidation is completely inhibited.

7. This inhibition is not due to the removal of catalytic metals, as with kaolin; for, on filtering off the tissue, solutions of reduced glutathione are obtained which are unable to react with oxygen (or methylene blue) even after the addition of iron or copper. They are, however, reactivated by the addition of a small amount of impure glutathione.

8. It is probable that the tissue inactivates the glutathione by removing the impurity which forms active complexes with metals. Free iron and copper are catalytically inactive in the oxidation of glutathione, and glutathione is unable to form active complexes with them. It follows that pure reduced glutathione is not autoxidisable, even in presence of iron or copper.

9. Other peptides of cysteine behave in a similar way to glutathione.

10. Certain other proteins behave like the "thermostable preparations" in inactivating glutathione. The oxidation of glutathione is also inhibited by fresh washed muscle.

11. Evidence is given that the catalytic substance, other than metals, concerned in the oxidation of glutathione can be formed, under certain conditions, from the glutathione molecule itself.

12. A mixture of crystalline glutathione and free cysteine behaves in the same way as the earlier glutathione preparations.

13. While the properties of the catalytic impurity in the glutathione preparations can be very closely imitated by free cysteine, quantitative experiments do not altogether support the view that this substance is cysteine itself.

14. In the oxidation of unsaturated fatty acids crystalline glutathione shows the same catalytic power as the earlier preparations.

We wish to acknowledge our indebtedness to Sir F. G. Hopkins, for his kind advice and criticism. One of us (N. U. M.) was in receipt of grants from the Carnegie Research Board and the Department of Scientific and Industrial Research.

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LVII. FURTHER EXPERIMENTS ON CANCER-PRODUCING SUBSTANCES.

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THE carcinogenic factor in gas-works tar is known to be present in the higher-boiling fractions. A list has been compiled [Kennaway, 1924, 1] of the compounds known to be present in tar with boiling points above 270°, which temperature appeared to indicate very roughly the boundary between the lower-boiling non-carcinogenic, and higher-boiling fractions. The list is reproduced below (Table I) together with the results obtained in this laboratory and others [Bloch, 1922; Twort and Fulton, 1930] by application of some of these compounds to mice.

Table I. *Substances in tar which boil above* 270°.

	Cook, Hieger and Kennaway	Bloch [1922]	Twort and Fulton [1930]
Acenaphthene	–	–	–
Fluorene	–	–	–
Fluoranthene	.	–	.
Anthracene	–	–	–
Dihydroanthracene	.	.	–
Hexahydroanthracene	.	.	.
β-Methylanthracene	–	–	–
Phenanthrene	–	–	–
Retene	–	–	–
Pyrene	under test	–	nil 35 weeks
Chrysene	–	–	+
Picene	–	.	.
Truxene	–	.	–
Naphthacene	–	.	.
Naphthanthracene	under test	.	.
Chrysogene	.	.	.
Benzerythrene	.	.	1 papilloma
Crackene	.	.	.
Diphenylene oxide	under test	.	.
β-Naphthofurane	.	.	.
α- and β-Naphthol	.	.	.
α-Dimethylquinoline	.	.	.
Carbazole	–	–	.
Phenyl-*p*-naphthylcarbazole	.	.	.
Acridine	–	.	.
Diphenylene sulphide	.	.	.

+ and – indicate positive and negative results of application to mice.

The table shows discordant results with chrysene. Chrysene (at first 1 %, then 0·33 % suspension of Kahlbaum's preparation in "90 % benzol" at

40°) was applied here to 100 mice for 11 months with negative results [Kennaway, 1924, 1], but only 5 of these were alive after 6½ months. Twort and Fulton [1930] report 5 cancers and 10 warts in mice painted with chrysene (suspension in oleic acid or liquid paraffin) either alone or with other compounds; apparently they used several hundreds of mice, but they do not state the exact number exposed to chrysene. From the yield of tumours they conclude that the carcinogenic potency of chrysene, in such suspensions, must be very low. The negative results with some of the above substances are not very satisfactory, owing to their very low solubility (*e.g.* naphthacene, truxene, picene). Naphthacene, with dihydronaphthacene, was prepared by Hieger by the method of Gabriel and Leupold [1898]; naphthanthracene, with dihydronaphthanthracene, was prepared by Cook by the method of Clar and John [1929]; in both cases the hydrocarbon and the partially reduced compound were applied together.

SYNTHETIC CARCINOGENIC MATERIALS.

I. *Tars.*

Various attempts have been made to obtain if possible carcinogenic material of a nature simpler than coal tar. Isoprene, acetylene, human skin, yeast, and cholesterol were heated between 700° and 920°, and cancer-producing mixtures, some of them very active, were obtained from each one of these [Kennaway, 1924, 2; 1925; Kennaway and Sampson, 1928]. Human voluntary muscle also, heated to 920° is very effectual (4 papillomata and 12 cancers from 100 mice in 243 days). By these methods extremely active tars have been prepared by Twort and Fulton [1930] from turpentine and from pinene.

II. *Products of the action of aluminium chloride upon tetralin.*

It was thought desirable to seek for carcinogenic compounds formed at lower temperatures. Accordingly the high-boiling mixture of compounds [Schroeter, 1920] obtained by the action of $AlCl_3$ upon tetrahydronaphthalene (tetralin) was examined; for the sake of brevity this mixture is spoken of here as "Schroeter." (1) 220 g. tetralin were left at 37° for 18 hours with 7·5 % by weight of $AlCl_3$, and then decomposed with water in the usual way. The oil was distilled over the free flame to remove tetralin (B.P. 204°) until the temperature in the neck was 270°; the clear viscid reddish-yellow residue was painted at first undiluted, later diluted with 2 vols. xylene, upon 30 mice. All were dead in 148 days, but one papilloma was produced, and the same material produced papillomata upon the ears of 2 rabbits. (2) A repetition of the experiment with two fresh batches of material prepared in the same way gave 11 tumours (8 cancers, 3 papillomata) in 20 mice in 256 days. (3) If a larger proportion of $AlCl_3$ (36 %) was allowed to act for a longer time (18 days) the product, applied at first undiluted and later diluted with an equal volume of benzene, was no more active (13 cancers and 6 papillomata from 86 mice in 292 days; the first tumour appeared on the 53rd day). (4) The residue from the action of 3 % $AlCl_3$ for 3 hours only, after distillation to 230° in the neck, gave no tumours. (5) The variability of commercial tetralin caused many difficulties; from some samples $AlCl_3$ produced little or no higher-boiling fraction. One of the most active preparations was made by Hieger from tetralin which he had converted into sulphonate and, after liberation, fractionated within very narrow limits (207·2° to 207·4°); this was then treated by Schroeter's method (2 % $AlCl_3$ for 9 hours at 65°) and then distilled at 15 mm. in nitrogen so as to give four fractions *a*, *b*, *c*, *d*, each of which was painted in solution in benzene on 50 mice. The results were as follows:

	Fraction	Papillomata	Epitheliomata
a.	Up to 205°	0	0
b.	205–260°	3	10
c.	260–320°	3	30
d.	Residue	2	0

Evidently there was a considerable concentration of active substance in (*c*); the fluorescence spectrum of this fraction has been used extensively for comparison (see following paper). From this, and from some other experiments in which undistilled material has given negative results, it seems probable that the carcinogenic substance is produced by the heating in distillation, or at any rate is so increased in amount by this heating that it is able to produce tumours in mice (on the minimum concentration of such substances needed to produce cancer see Hieger [1929]). Hence the original object of these experiments, namely to produce carcinogenic substances at body temperature, may not have been attained. (6) Five similar series of experiments in which naphthalene, in place of tetralin, was acted on by $AlCl_3$ gave only 1 papilloma and 1 cancer from 130 mice.

The total yield of tumours produced by suitably prepared "Schroeter" has been 118 cancers and 30 papillomata from 496 mice. Further chemical and spectroscopic experiments are in progress upon the products from a very pure tetralin made by the staff of Technical Research Works, Ltd., to whom we are indebted for the great amount of care devoted to the preparation.

Schroeter [1924] found in the products of the action of $AlCl_3$ (2 %) upon tetralin (6–10 hours at 50–70°) benzene, octahydroanthracene (octhracene), octahydrophenanthrene (octanthrene), α-phenyl-δ-2-tetralylbutane, and 2 : 6′-ditetralyl (the last two identified in the oxidised forms as α-phenyl-δ-naphthylbutane and 2 : 2′-dinaphthyl respectively). By the further action of $AlCl_3$ upon octhracene, he obtained material yielding octanthrene, tetralin, and dodecahydrotriphenylene; from this he infers the production of some compounds (phenylene-*bis*-octhracenylbutane, tetralyloctanthrenylbutane) not isolated.

As a control upon the results obtained with "Schroeter," 30 mice were painted with untreated commercial tetralin; from these, one epithelioma was obtained on the 132nd day. 100 mice were then painted with tetralin which had been purified by treatment with $NaHSO_3$ followed by rectification between 204° and 206°; these yielded no tumours. Commercial tetralin may contain as much as 20 % of decalin; 10 mice which were painted with commercial decalin gave only a subcutaneous melanoma such as has occurred in various other series [cf. Twort and Twort, 1928], but no epithelial tumours. Dialin likewise gave negative results. The question whether the one cancer produced by commercial tetralin was due to an impurity, or to a special susceptibility of the mouse, must remain uncertain.

III. *Products of the action of aluminium chloride upon acetylene, xylene, and other substances.*

Hieger (unpublished observations) condensed acetylene by treatment with aluminium chloride at room temperature [Baud, 1900] and painted the non-volatile products, extracted in a Soxhlet with boiling benzene, upon mice. The difficulty of obtaining the material in quantity allowed of the painting of 30 mice only, among which 1 epithelioma and 1 cystic tumour of doubtful nature occurred. Thus acetylene yields carcinogenic products both (*a*) when passed through a red-hot tube, and (*b*) when acted upon by $AlCl_3$ with exposure to no temperature higher than the boiling point of benzene.

The products of the action of $AlCl_3$ upon various other substances (xylene, cholesterol, lanolin, sebaceous material from dermoids, human skin), of which some had given carcinogenic tars, were tested upon mice. All these gave negative results except the preparation from xylene, which yielded 1 papilloma and 1 cancer in 100 mice, and a papilloma upon the ear of a rabbit. High-boiling compounds, including apparently methylanthracenes, have been obtained by the action of $AlCl_3$ on xylene [Anschütz, 1886; Moore and Egloff, 1917].

COMPOUNDS RELATED TO NAPHTHALENE.

In view of the ease with which carcinogenic substances are obtained from tetralin, various compounds derived from, or closely related to, naphthalene were tested (Table II); all gave negative results. Most of the substances named in Tables II and III were applied in benzene. Many of the compounds of low molecular weight (*e.g.* phenyl*iso*crotonic acid, cyclohexene) named in Tables II and III were taken, not with any expectation that they were themselves carcinogenic, but with the idea that they might serve as materials for synthesis in the skin of the mouse; this idea being suggested especially by the instance mentioned above, where an epithelioma arose in a mouse painted with tetralin only.

Table II.

1. **Naphthalene.**
2. **Naphthoylbenzoic acid.**
3. **β-Naphthoic acid.**
4. **Dinaphthylene dioxide.**
5. **β-Dinaphthyl ether.**
6. **ββ-Dinaphthyl.**
7. **αα-Dinaphthyl.**
8. **α-Phenylnaphthalene.**
9. **β-2-Tetroylpropionic acid.**
10. **γ-2-Tetralylbutyric acid.**
11. **Phenyl*iso*crotonic acid.**

Dinaphthylene dioxide was prepared because it has been stated to be an impurity which imparts the yellow colour to chrysene; the evidence for this seems quite insufficient, but the matter was too important to be left untested. In addition to the two dinaphthyls, crude reduction products (made by treatment with hydriodic acid and phosphorus) from both were tested in view of the presence of $\beta\beta$-ditetralyl in "Schroeter." (8) was prepared by Hieger by the action of $AlCl_3$ upon C_6H_5Br and $C_{10}H_8$ [Chattaway, 1893]. The distillates up to 342° containing the hydrocarbon were inactive, but the residue in the flask was strongly carcinogenic (6 cancers and 3 papillomata from 30 mice in 279 days); possibly a pyrogenous reaction had occurred producing compounds allied to those in "Schroeter." (9) gave no tumours, but the uncrystallisable oil from which it separates produced in 3 mice out of 20 cystic dilatations of hair follicles of doubtful nature; the acid is prepared [Krollpfeiffer and Schäfer, 1923] by the action of $AlCl_3$ upon tetralin and succinic anhydride, and hence may be accompanied by some compounds similar to those present in "Schroeter."

OTHER HYDROCARBONS AND QUINONES.

The following hydrocarbons, in addition to those named in the preceding tables, and quinones have been tested on mice.

Table III.

1. Cyclohexane.
2. Cyclohexene.
3. Styrene.
4. Hydrophenanthrene.
5. Hydrochrysene.
6. Hydroretene.
7. Dodecahydrotriphenylene.
8. Octhracene.
9. Octhracenone.
10. 1 : 2-Benzanthracene.
11. 1 : 2-Benzanthraquinone.
12. 2 : 3-Tetrahydrobenzanthracene.
13. Perylene.

All the compounds named in Table III, except (10), gave negative results. (2) is extremely irritating to the skin, and should produce cancer if "chronic irritation" in general were an essential factor in carcinogenesis. The three reduced hydrocarbons (4), (5) and (6) were made by the methods of Schmidt and Mezger, and Liebermann and Spiegel, which are stated to give an octahydrophenanthrene $C_{14}H_{18}$, a hydroretene $C_{18}H_{30}$, and a perhydrochrysene $C_{18}H_{30}$; none of these was isolated in the pure state. (8), which is present in "Schroeter," and its quinone were made by the method of Krollpfeiffer and Schäfer [1923]. In addition to (7), the mixture of high-boiling compounds remaining when diphenyl is prepared from benzene and distilled off in the ordinary way, which is stated to contain benzerythrene and triphenylene, was tested with negative result. Diphenyl itself does not produce tumours (Bloch). Twort and Fulton report negative results at the time of writing with triphenylene and dodecahydrotriphenylene. In a mouse of the benzanthracene series (50 mice) a well-developed papilloma appeared, which however was only transitory; but in the light of the subsequent results with dibenzanthracenes this was probably a significant observation. Perylene (for which, together with the phenanthrene-9-carboxylic acid required to make (10) in Table IV, we are indebted to the British Dyestuffs Corporation) is still under test.

The following hydrocarbons were synthesised by the methods of Clar and his fellow workers [1929] and of Fieser and Dietz [1929], and applied to mice, 10 to 20 mice being used for each substance. The selection of these compounds for special study was due to the spectroscopic work of Hieger, which is described in the following paper.

Table IV.

	Hydrocarbon	Solution
1.	1 : 2 : 5 : 6-Dibenzanthracene.	Saturated, in benzene (less than 1 %)
2.	1 : 2 : 7 : 8-Dibenzanthracene	Saturated, in benzene (less than 2 %)
3.	1 : 2 : 3 : 4-Dibenzanthracene	Saturated, in benzene (less than 1 %)
4.	Mixture of isomers of (3), probably naphtho-2′ : 3′ : 1 : 2-phenanthrene and naphtho-2′ : 3′ : 2 : 3-phenanthrene	Nearly saturated, in benzene
5.	Naphtho-2′ : 3′ : 1 : 2-anthracene	1 % in benzene
6.	7 : 7′-Dimethyl-(naphtho-2′ : 3′ : 1 : 2-anthracene)	1 % in benzene
7.	Anthraceno-2′ : 1′ : 1 : 2-anthracene	Saturated, in xylene
8.	Anthraceno-1′ : 2′ : 1 : 2-anthracene	Saturated, in xylene
9.	3′-Methyl-1 : 2 : 5 : 6-dibenzanthracene	Saturated, in benzene (less than 0·5 %)
10.	1 : 2 : 3 : 4 : 5 : 6-Tribenzanthracene	0·3 % in benzene
11.	2 : 3 : 8 : 9-Di-[naphtho-1 : 2′]-chrysene	Suspensions in tetralin and in oleic acid

Most of these were not highly purified, as this may require as many as twenty recrystallisations (Clar) involving considerable loss; but the preparations used should suffice to show whether the hydrocarbon in question is not carcinogenic. All the syntheses except that of (5), which involves a Grignard reaction, depend upon the action of $AlCl_3$ to produce the intermediate ketone. (10) and (11) were prepared by J. W. Cook. The compounds, except (7), (8) and (11), were dissolved in benzene for painting; the concentration was not in all cases the same but was dependent upon the amount available, and upon the solubility, of the compound. Sometimes the mother-liquor of the final recrystallisation was used, to economise material. (7) and (8) are very little soluble in lower boiling solvents, and were applied as the dilute saturated solutions in xylene; this is an undesirable solvent, as it produces considerable irritation and roughness, and occasional transient papillomata, in the skin of the mouse. (11), which is still more insoluble is applied in suspension in tetralin and in oleic acid. Some of these substances have at the time of writing (February, 1930) been applied to mice for a short time only.

10 mice were painted with (2). A mouse which died on the 203rd day showed changes suggesting the early stages of a papilloma. On the 169th day, one of the 6 mice surviving bore a papilloma; the animal was killed on the 223rd day, and the tumour was found to be clearly malignant, showing invasion of muscle over a wide area. Another mouse showed a papilloma on the 222nd day; the tumour grew rapidly, and when the mouse was killed a month later showed obvious invasion of muscle. At the date of writing (252nd day),

of the 2 surviving mice, one bears a papilloma, the other shows no tumour. Thus 2 cancers and 1 papilloma have been obtained in the series of 10 mice. 10 mice were painted with (1). On the 232nd day, 1 of the 3 mice surviving bore a well-developed papilloma which is growing at the present date; none of the others shows a tumour. (1) and (2) are almost identical in some physical properties, differing by about 1° in melting point.

The preparation of (2) used for painting should be fairly pure, being obtained from the mother liquor of crystals giving a very sharp melting point (260–260·5°). All such results of course require confirmation with very pure material. The sample of (1) applied to mice was less pure than that of (2). These are the only instances in all the experiments described in this paper in which, as yet, cancers have been produced by a preparation of a fairly high degree of purity, for commercial tetralin, which caused one tumour, certainly could not be described in this way. The dibenzanthracenes can be only weak carcinogenic agents, for the incidence of tumours is low and the precancerous period long, but the result is an indication for further experiments, which are in progress[1]. Apparently neither benzanthracene nor any of its derivatives have been found, and perhaps have not been sought for, in coal tar, and we are at present examining tar, and the products of the action of $AlCl_3$ upon tetralin, for such compounds. It is quite possible that there are, among the many compounds still undiscovered in coal tar, derivatives of benzanthracene or of chrysene which are far more powerfully carcinogenic than any known substances.

A number of new hydrocarbons (1-phenylanthracene, 2-phenylanthracene, 3-methyl-1 : 2-benzanthracene, 10-benzyl-1 : 2-benzanthracene, 6-phenyl-1 : 2-benzanthracene, 9 : 10-diphenyl-1 : 2-benzanthracene, acenaphthanthracene, 10-benzylacenaphthanthracene, 4′-methyl-1 : 2 : 7 : 8-dibenzanthracene, 2 : 3-benzo-8 : 9-[1′ : 2′-naphtho]-chrysene) have been synthesised by J. W. Cook in this laboratory, by methods which he will describe in the *Journal of the Chemical Society*; all these compounds are being tested on mice.

SUMMARY.

1. Carcinogenic products have been obtained by the action of aluminium chloride upon (*a*) acetylene, (*b*) xylene, (*c*) naphthalene, (*d*) naphthalene and bromobenzene, and (*e*) tetrahydronaphthalene. Of these (*d*) and (*e*), under the conditions described, give the most active materials.

2. Two cancers have been obtained in a series of 10 mice painted with a solution of a fairly pure specimen of 1 : 2 : 7 : 8-dibenzanthracene.

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[1] (*Note added at correction of proof.*) Two more cancers have been obtained from the mice of the 1:2:7:8-dibenzanthracene series; thus the last four of the 10 mice painted with this material have all given cancers. The experiments will be repeated with a sample of the hydrocarbon freed completely from yellow colouring matter. The last four of the 10 mice painted with impure 3′-methyl-1:2:5:6-dibenzanthracene have developed papillomata.

I wish to express my indebtedness to the British Empire Cancer Campaign, whose grants to I. Hieger have enabled him to develop methods which have been essential to the experiments described here. Dr E. de Barry Barnett has most kindly given many valuable specimens of pure hydrocarbons for use in this laboratory. It is a pleasure to record the very large part that has been played throughout these investigations by the work of my assistant F. Goulden.

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LVIII. THE SPECTRA OF CANCER-PRODUCING TARS AND OILS AND OF RELATED SUBSTANCES.

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This work deals with the application of optical methods to the identification of the cancer-producing agent present in tars and oils. The chemical separation of the cancer-producer will prove difficult since tars and oils are exceedingly complex mixtures, but the search has been narrowed by various investigations (see references in preceding paper) which suggest that the active agent is among the aromatic hydrocarbons with condensed rings. These compounds, represented by anthracene, possess the property of fluorescing intensely when exposed to ultra-violet light and the results and opinions expressed here are based on an examination of the fluorescence spectra and ultra-violet absorption spectra of several hundred substances, in part pure compounds, in part mixtures.

EXPERIMENTAL.

A quartz mercury vapour lamp (Kelvin, Bottomley and Baird) was used as a source of ultra-violet light. The lamp stood in an asbestos box in one side of which was a Wood's glass window enclosed by a fish-tail shaped tube, 3 cm. in diameter. Only filtered ultra-violet light emerged from the flattened aperture of the tube. The solutions were examined in corked tubes of non-fluorescing "monax" glass 70 × 6 mm. and of egg-shell thickness. The tube was clamped directly in front of the fish-tail aperture so as to prevent almost completely the escape of the exciting radiation. The spectroscope (Hilger, no. E 31) was placed with its slit about 1 cm. from the fluorescence tube and the axis of the collimator tube made an angle of about 80° with the normal of the incident rays. Such an arrangement reduced the tendency for the reception of the spectrum of the incident light reflected from the surface of the glass tube. The best position for the spectroscope was found by removing the plate holder and placing the eye in the focal plane and at a point corresponding with the focussed image of the visible part of the spectrum. A disc of bluish-green fluorescing light suddenly comes into view when the spectroscope is very slowly rotated about its central axis, indicating the optimal angle. For this sort of photography the extremely fast panchromatic plates now available possess no advantage over the panchromatic plates of ordinary speed. Correct exposure, determined by trial, varied from 5 seconds to an hour or more.

It is often difficult to avoid photographing the reflected spectrum of the incident light especially when the exposure is prolonged; the reflections appear as curious parabolic blurs, or as multiple fine lines, or as heavy fogging in the short wave-length region of the spectra.

Specificity of fluorescence spectra.

Fluorescence spectra do not distinguish between some of the aliphatic *meso*-derivatives of anthracene; 9-methylanthracene and 9-benzylanthracene give almost identical spectra. On the other hand the spectra of $\alpha\alpha$-dinaphthyl and $\beta\beta$-dinaphthyl can be easily distinguished. This experiment indicates the possibility of identifying closely related isomers. Commercial diphenylmethane is brightly fluorescent when appropriately excited, and its spectrum is identical with that of anthracene, but if diphenylmethane is synthesised in the laboratory the fluorescence is so faint that a 20 % solution of this hydrocarbon is as weakly fluorescent as a 0·002 % solution of anthracene. Such an experiment illustrates the use of a method of spectrum analysis in the detection of traces of highly fluorescent substances in the presence of large quantities of others not, or weakly, fluorescent.

Effect of substitution on fluorescence spectra.

When anthracene is substituted in the α- or β-position by Cl or CH_3 there is a shift of the spectrum in the direction of longer wave-length, the relative position of the bands and their general appearance remaining unchanged. But a tetramethylanthracene shows a spectrum only slightly different from that of the monomethyl derivative. This instance lends no support to any idea of proportionality between the amounts of substitution and of spectrum shift.

When the *meso*-hydrogens of anthracene are substituted by aliphatic groups like methyl or benzyl the spectrum resembles the ordinary three-banded anthracene spectrum, but in the spectrum of *meso*-phenyl- and *meso*-naphthylanthracene the resemblance falls off and in *meso*-diphenylanthracene is gone. Benzene has been shown to possess fluorescence bands themselves situated in the ultra-violet region, and on general principles it may be justifiable to conclude that the curious spectrum of 9 : 10-diphenylanthracene is in part, at least, due to its behaviour as a compound which can be regarded as anthracene-substituted benzene. Some of the methylated polybenzanthracenes give fluorescence spectra identical with that of the parent polybenzanthracene. One of these is represented in Plate III (no. 278, 1 : 2 : 5 : 6-dibenzanthracene). Probably the disproportion in relative mass of the small methyl group and the large dibenzanthracene nucleus accounts for the stability of the spectrum.

Effect of concentration, solvent, and irradiation.

Strong and weak solutions of 9 : 10-dibenzylanthracene and 9 : 10-dimethylanthracene give slightly different spectra, but this effect has not been observed with varying dilutions of other compounds where the concentrations were chosen at random. The concentration effect observed with these two anthracene derivatives is perhaps the result of a swamping action of one band upon another connected with their remarkably powerful fluorescence; one part in two hundred million of solvent still shows fluorescence.

Whether the solvent has any influence on the nature of the fluorescence has been tested in only one case. Solutions of $AlCl_3$-treated tetralin in chloroform, light petroleum, and alcohol gave identical spectra.

When the chloroform solution of $AlCl_3$-treated tetralin was exposed to ultra-violet light for more than about 30 minutes, the beautiful violet-blue fluorescence slowly changed to a pale grey-blue. Solutions of the tetralin preparation in light petroleum and alcohol can be irradiated for 3 hours without the least change in the original colour or spectrum. Fading effects are also observed with xylene solutions of stilbene and with a chloroform solution of a mixture of naphthacene and dihydronaphthacene. The fading of chloroform solutions led to the use of light petroleum as a more inert solvent and later to the use of benzene, because of the insolubility in light petroleum of some of the more complex hydrocarbons.

Spectra of cancer-producing substances, and especially of that obtained from tetralin.

Among the first few substances to be examined were some cancer-producing tars and oils, a number of which show similar bands (see Plate III). The bands were in position 4000, 4180, 4400 Å., sharply defined on the short wave-length side, and grading less abruptly into the background on the long wave-length side. Such graded bands will be referred to as "fluted."

For purposes of comparison, we have found it necessary to use a pair of dividers to contrast or compare spectrum bands on the paper photographs with one another or with the standard 4000, 4180, 4400 Å. bands or with the benzanthracene bands, the importance of which is discussed later. We have used the very strong 3680 Hg line as a suitable fixed wave-length from which measurements can be made. In Plates III and IV the photographs have been so arranged that the 3680 Hg lines of the spectra are in alignment.

For the comparison of spectra on glass plates, the method used is simply to superimpose a test plate on the standard 4000, 4180, 4400 spectrum plate, or on the standard benzanthracene spectrum plate. The five variables consist of number and nature (fluting) of bands, absolute wave-length of bands, their position relative to one another and their individual intensity.

An interesting cancer-producing substance is that obtained as the high-boiling fraction from the mixture resulting from the action of $AlCl_3$ on commercial tetrahydronaphthalene (tetralin). This type of reaction and the study of the products as carcinogenic agents are described in the preceding paper. Tetrahydronaphthalene is allowed to react with 2 % $AlCl_3$ for 9 hours at 65°, the crude mixture decomposed with water, dried and fractionated. The fraction distilling at 260–320° at 14 mm. is a powerful cancer-producing agent even in concentration of only 4 %. It is a clear viscid syrup, yellow or orange in colour, and intensely fluorescent; one part in 40 millions of ether can still be distinguished from pure ether. The fluorescence spectrum is very sharply defined and identical with the spectrum so frequently found in cancer-producing tars and oils. One or two of the numerous subsequent batches of the 260–320° fraction did not give spectra exactly like the standard. One of these aberrant spectra was given by a fraction made from tetralin, prepared by Technical Research Works, Ltd., by a method which ensures that the final product is free from decalin; but the usual 4000, 4180, 4400 Å. spectrum was obtained from the 260–320° fraction prepared from pure tetralin made by another method, namely the sulphonate separation from commercial tetralin. The former of these preparations is under test on mice; the latter was carcinogenic.

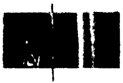
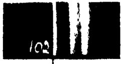
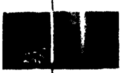
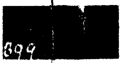
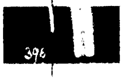
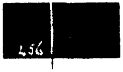
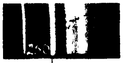
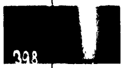
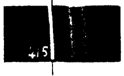
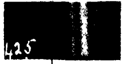
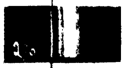
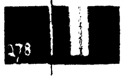
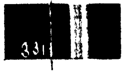
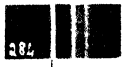
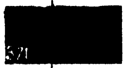
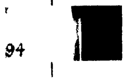
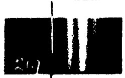
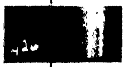
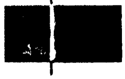
The possibility must be considered that the substance responsible for the bands may not be identical with the cancer-producer but is some closely allied compound, frequently, if not invariably, produced when the cancer-producer is formed, either by low temperature catalysis or at high temperature. The carcinogenic agent in tars and oils may be only one of a group of such compounds.

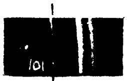
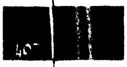
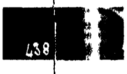
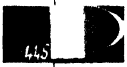
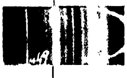
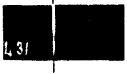
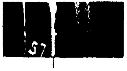
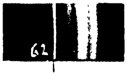
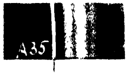
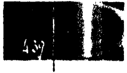
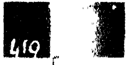
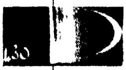
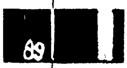
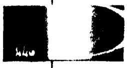
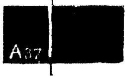
The very frequent recurrence of the 4000, 4180, 4400 Å. bands in carcinogenic agents and the delicacy and class specificity (see previous section and Plates III and IV) of the fluorescence test justify the use of the fluorescence spectrum to indicate the probability of carcinogenic activity and to assist in directing the preparation of appropriate hydrocarbons. The test on mice is, of course, necessary for conclusive proof.

The fluted bands (4000, 4180, 4400 Å.) are shown more distinctly by $AlCl_3$-treated tetralin [see Kennaway, 1930] than by any other cancer-producing substance. Now very similar bands are given by 1 : 2-benzanthracene; these bands also are strongly fluted and only in their absolute position in the wave-length scale do they differ from the 4000, 4180, 4400 Å. bands.

On generally accepted grounds one would expect that it might be possible, by increasing the mass of the benzanthracene molecule by suitable substitution, to produce a compound with a corresponding shift of the spectrum in the direction of increased wave-length possessing a spectrum with bands at 4000, 4180, 4400 Å. To this end a number of benzanthracene derivatives have been made in this laboratory; many of the new compounds have been synthesised by Dr J. W. Cook (see Plate III). A compound with a fluorescence spectrum intermediate between those of 1 : 2-benzanthracene and $AlCl_3$-treated tetralin, namely, 1 : 2 : 7 : 8-dibenzanthracene, has yielded 2 cancers in 10 mice.

A spectrum having bands at 4000, 4180, 4400 Å., has been obtained from the following cancer-producing materials (Plate IV).

	Substance	Carcinogenic activity	Fluorescence spectrum
101	$AlCl_3$-treated tetralin	Active	
102	1 : 2-Benzanthracene	1/50	
225	4-Chloro-1 : 2-benzanthracene	Not tested	
399	3-Methyl-1 : 2-benzanthracene	Under test	
396	6-Phenyl-1 : 2-benzanthracene	Under test	
456	6-*iso*Propyl-1 : 2-benzanthracene	Under test	
250	6-Methyl-1 : 2-benzanthracene	Under test	
398	9 : 10-Diphenyl-1 : 2-benzanthracene	Under test	
415	10-Benzyl-1 : 2-benzanthracene	Under test	
425	Tetrahydro-1 : 2-benzanthracene	Under test	
276	1 : 2 : 3 : 4-Dibenzanthracene	Under test	
278	1 : 2 : 5 : 6-Dibenzanthracene	1/10	
331	3′-Methyl-1 : 2 : 5 : 6-dibenzanthracene	4/10	
281	1 : 2 : 7 : 8-Dibenzanthracene	4/10	
371	1 : 2 : 3 : 4 : 5 : 6-Tribenzanthracene	Under test	
94	Naphthacene-dihydronaphthacene	Inactive	
219	Tetrahydro-2 : 3-benzanthracene	Under test	
426	Dihydro*lin*dinaphthanthracene	Under test	
A64	Acenaphthene	Inactive	

	Substance	Carcinogenic activity	Fluorescence spectrum
101	$AlCl_3$-treated tetralin	Active	
407	Acenaphthanthracene	Under test	
438	10-Benzylacenaphthanthracene	Under test	
445	Chrysene	? See text	
449	Benzonaphthochrysene	Under test	
431	Dinaphthochrysene	Under test	
57	Picene	Inactive, see text	
62	Anthracene	Inactive	
A35	Phenanthrene	Inactive	
439	2-Phenylanthracene	Under test	
419	Triphenylene	? See text	
430	Pyrene	Under test	
89	Perylene	Under test	
446	ββ-Dinaphthyl	Inactive	
A37	Fluorene	Inactive	

The fractions in column 3 represent the ratio of tumour-bearing mice to total mice tested.

Table I.

A62‴	Gas works tar made at 1250°
A62′	Gas works tar made at 450°
85	Low temperature tar, 350°
A48	Creosote oil
A22	Green oil
A32	Anthracene oil
A21	Pitch distillate
A19	Heated Californian petroleum
A4	Acetylene tar
A72	Yeast tar
A73	Muscle tar
A25	Cholesterol tar
A23	Hair tar
101	$AlCl_3$-treated tetralin
100	1 % gas works tar
A3	Ether extract of gas works tar
A8	$AlCl_3$-treated naphthalene
244	Residue from preparation of α-phenylnaphthalene

One of the weakest of these agents was the tar made from human hair, which produced 2 cancers in 94 mice. The ether extract of gas works tar and a 1 % dilution of this (A3 and 100) were used in a quantitative study of the effect of dilution on cancer-producing activity [Hieger, 1929]. 101 is the "Schroeter" described in the preceding paper. The bands in A8 are extremely faint, and the material was feebly carcinogenic, producing 1 cancer in 50 mice. 244 requires special mention. It was strongly carcinogenic and gives the bands at 4000, 4180 and 4400 Å., together with another band of shorter wave-length.

The following materials gave a diffuse spectrum covering very roughly the whole area 4000–4400 Å. without differentiation into bands (Table II and Plate IV).

Table II.

A14	Shale oil (blue oil)
A16	Refined lubricating oil
A18	Blast furnace tar
A31	Unheated Californian petroleum
A50	Pitch (medium soft)
A62″	Gas works tar 600°
86	Spindle oil

Of these A14, A50, A62″ and 86 are carcinogenic. A16 and A18 were inactive. A31 yields when heated a strongly carcinogenic material A19 (Table I).

Only one material giving no bands in the region in question has produced a tumour, namely commercial tetralin (A6) which gave 1 cancer in 30 mice (see preceding paper).

The 32 hydrocarbons of which the fluorescence spectra are shown in Plate III fall into three classes.

(1) Those, 20 in number, which are still under test for carcinogenic activity.

(2) Those which have hitherto been found to be non-carcinogenic (see Table I in preceding paper); namely, anthracene, phenanthrene, fluorene, acenaphthene, pyrene, triphenylene, ββ-dinaphthyl, naphthacene and dihydronaphthacene. None of these gives the bands shown by the carcinogenic substances in Table I. Picene (a pure specimen obtained through the kindness of Dr Chattaway) gives two bands with rather diffuse edges, and of shorter

wave-length than 4000 and 4180 Å. It was tested here on 30 mice in suspension in dermoid oil; further tests will be made with it in other media.

(3) Those which have given tumours. Chrysene (445) which has produced cancers and papillomata in the experiments of Twort and Fulton [1930], but not in this laboratory, gives a spectrum very similar to that of 1 : 2-benzanthrene, but displaced considerably towards the region of shorter wave-length. 1 : 2-Benzanthracene gave 1 transient papilloma in 50 mice. 1 : 2 : 7 : 8-Dibenzanthracene has given 2 cancers in 10 mice[1]; it is noteworthy that the spectral bands of this compound lie about half-way between those of 1 : 2-benzanthracene and those of $AlCl_3$-treated tetralin and the other carcinogenic materials.

The following materials gave the same spectrum (Plate IV) as those in Table I, but did not produce cancer; β-tetroylpropionic acid mother-liquor (A26), undistilled $AlCl_3$-treated tetralin (A100), benzyl oleate (60), oleic acid (207). One may suggest two possible explanations for these discrepancies. (1) That the effect of the $AlCl_3$ on tetralin is only to prepare an intermediate substance which is rendered carcinogenic during distillation. It would be interesting to see if tetralin heated (under pressure) to the temperature of distillation of the $AlCl_3$-treated mixture became carcinogenic. It is conceivable that the spectrum (bands at 4000, 4180, 4400 Å.) is produced by the precursor of the carcinogenic agent. (2) Since many of the derivatives of anthracene remain fluorescent when diluted to one part in 200 millions of solvent it is possible that the minute quantity of active substance present may be sufficient for the fluorescence test yet be well below the carcinogenic threshold. Benzyl oleate gave no tumours in an experiment on 20 mice, although Twort and Twort [1928] obtained several papillomata in mice with oleic acid.

Absorption spectra.

$AlCl_3$-treated tetralin showed two bands at about 3700 and 3900 Å. and between these limits of wave-length the curve exactly followed the absorption curve of benzanthracene.

SUMMARY.

The fluorescence spectra of many cancer-producing substances show the same bands at wave-lengths 4000, 4180, 4400 Å. These bands are remarkably like those of the fluorescence spectrum of 1 : 2-benzanthracene, apart from their absolute wave-length. A study of the fluorescence spectra of a large number of condensed ring aromatic hydrocarbons shows that the spectrum is characteristic for closely related derivatives of a hydrocarbon. Absorption

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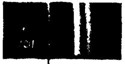
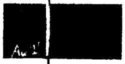
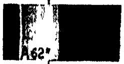
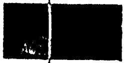
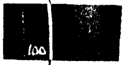
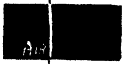
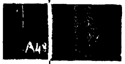
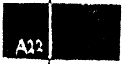
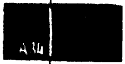
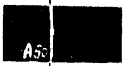
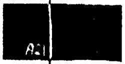
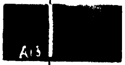
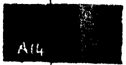
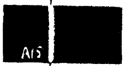
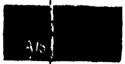
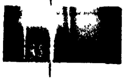
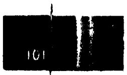
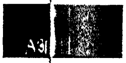
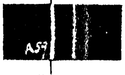
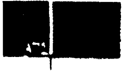
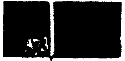
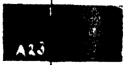
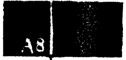
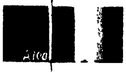
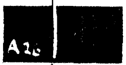
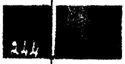
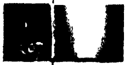
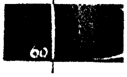
[1] (*Note added at correction of proof.*) Two more cancers have been obtained from the mice of the 1:2:7:8-dibenzanthracene series; thus 4 of the 10 mice painted with this material have given cancers. Four of the 10 mice painted with impure 3′-methyl-1:2:5:6-dibenzanthracene have developed papillomata and papillomata have been obtained in 1 mouse from the series of 10 painted with 1:2:5:6-dibenzanthracene.

	Substance	Carcinogenic activity	Fluorescence spectrum
101	$AlCl_3$-treated tetralin	Active	
A62	Durham Holmside gasworks tar 450	Active	
A62''	Durham Holmside gasworks tar 600	Active	
A62'''	Durham Holmside gasworks tar 1250°	Active	
A3	Ether extract of gasworks tar	Active	
100	1 % gasworks tar	Active	
A18	Blast furnace tar	Inactive	
A48	Creosote oil	Active	
A32	Anthracene oil	Active	
A22	Green oil	Active	
A34	Anthracene 85 %	?	
A50	Pitch (medium soft)	Active	
A21	Pitch distillate	Active	
141	Pitch distillate 300–345° 1 mm.	Under test	
A13	Shale oil (Pumpherston S)	Inactive	
A14	Shale oil (blue oil)	Active	
A15	Shale oil (Pumpherston R)	Inactive	
A16	Refined lubricating oil	Inactive	
85	Low temperature tar 350°	Active	

	Substance	Carcinogenic activity	Fluorescence spectrum
101	$AlCl_3$-treated tetralin	Active	
86	Spindle oil	Active	
A31	Unheated Californian petroleum	Inactive	
A19	Heated Californian petroleum 880°	Active	
A4	Acetylene tar	Active	
A59	Chlorinated acetylene tar	Active	
A72	Yeast tar	Active	
A73	Muscle tar	Active	
A23	Hair tar (800°)	2/91	
A25	Cholesterol tar	Active	
A8	$AlCl_3$-treated naphthalene	1/50	
A100	Undistilled $AlCl_3$-treated tetralin	Inactive	
A6	Commercial tetralin	1/30	
A26	Tetroyl-propionic acid mother liq.	Inactive	
244	Residue from preparation of phenylnaphthalene	Active	
207	Oleic acid ("redistilled")	Inactive	
60	Benzyl oleate	Inactive	

The fractions in column 3 represent the ratio of tumour-bearing mice to total mice tested.

spectra in the ultra-violet also indicate, so far as the experiments have been carried, some connection between at least one powerfully cancer-producing material (*i.e.* the fraction boiling at 260–320°/14 mm. from the mixture obtained by the action of $AlCl_3$ on commercial tetrahydronaphthalene) and 1 : 2-benzanthracene.

The author is indebted to the British Empire Cancer Campaign for a research grant; to Prof. Leitch, the Director of this Institute, for the use of its facilities; to Dr Kennaway, who supervised the animal experiments; to W. V. Mayneord, who photographed many of the spectra; to Dr E. de Barry Barnett, for the loan of specimens of pure hydrocarbons; and to Dr J. W. Cook for a number of pure synthetic compounds.

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—— C. C. and Twort, J. M. (1928). *J. Hyg.* **28**, 219.

LIX. OXIDATION-REDUCTION POTENTIALS OF CULTURES OF HAEMOLYTIC STREPTOCOCCI. I.

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(Received March 4th, 1930.)

Haemolytic streptococci can multiply over a wide range of conditions of oxygen supply from almost complete anaerobiosis to full exposure to the air, as on the surface of an agar slope. This ready adaptation to different conditions must involve changes in metabolic activity and biological behaviour. The measurement of oxidation-reduction potentials is the most direct method of following some of the effects of environmental conditions on the metabolic processes of the organisms.

When an "unattackable electrode" is immersed in a solution containing a reversible oxidation-reduction system a potential difference is set up at the electrode, and this potential may be shown, by simple thermodynamic reasoning, to be dependent on the proportion of oxidised and reduced forms of the substance studied. The potential of the normal hydrogen electrode is taken as the standard of reference and to this is ascribed the value of zero potential difference. An electrode potential referred to this standard is designated E_h and is measured in volts. The higher the oxidising intensity of a system the more highly positive is the E_h, and the more highly reducing a system the more negative is the E_h. An essential point to be remembered is that electrode potential, like temperature and p_H, is a measure of intensity level and not of capacity.

Bacterial cultures develop reducing tendencies during growth as shown, for example, by the decoloration of methylene blue. It would seem therefore that oxidation-reduction indicators might be employed for the determination of oxidation-reduction potential, in the way that p_H indicators are used for the determination of hydrogen ions. There are, however, certain disadvantages in the use of oxidation-reduction indicators, particularly in bacterial cultures. One disadvantage is that the oxidising capacity of the indicator is frequently great compared with the reducing capacity of the system, so that the bacterial culture is oxidised by the dye and the equilibrium is thereby displaced. Another disadvantage is the time taken for the dye to come into equilibrium with the system, which, in the case of bacterial cultures, is itself constantly changing. A further disadvantage is the participation of the dye in the system and its possible toxic action on the organisms. Continuous readings cannot be made with a toxic dye; moreover, the killed organisms may have a different effect on the electrode potential of the medium. It is considered essential therefore to make direct determinations of the electrode potentials of bacterial cultures.

Gillespie [1920], who was particularly interested in the behaviour of waterlogged soils, first noted the steady drift towards negative potentials in bacterial cultures. This was confirmed by Cannan, Cohen and Clark [1926], and by Fildes [1929] who found that a certain limiting reduction potential is necessary for the germination of tetanus spores.

The present communication is the outcome of some work carried out with Dr E. W. Todd in connection with the biological behaviour of haemolytic streptococci under different cultural conditions. The purely bacteriological aspect of the subject will be dealt with by Dr Todd in a communication shortly to be published.

The conditions of cultivation were found to have a marked effect on the electrode potential-time curves of the cultures and the results obtained are reported with the minimum of comment until it is felt that chemical interpretations and biological implications are substantiated.

Methods.

The oxidation-reduction potentials of bacterial cultures are generally not well poised, so that it is essential to avoid polarisation effects. For this reason and for rapidity of measurements the Lindemann electrometer proved an excellent instrument for determining the null-point of potentiometric balance. Measurements were facilitated by the use of a series of single-pole, double-throw knife switches for connecting each individual cell and a double-pole, double-throw switch for reversing the connections to the potentiometer.

Glass double-limbed cells placed in wooden blocks were used, and, in some experiments, the cultures were kept in a state of agitation and aeration by using the three-limbed tube shown on the left in Fig. 1. The central tube was connected by rubber tubing to Ridley's [1928] mixing apparatus. In this way the cultures were alternately drawn up and depressed in the central tube, both the rate of movement and extent of excursion being readily adjusted. An agar-KCl bridge dipped into one limb of the culture-cell and into a vessel containing saturated KCl solution into which also a $3{\cdot}5N$ KCl-calomel electrode dipped. Both gold wire and gold foil electrodes proved unreliable, so that platinum wires sealed into glass tubing were used and these gave steady reproducible readings. Some irregular results were explained by the discovery of a cracked electrode which caused direct electrical contact of mercury with the culture medium. Eight cells and the calomel electrode were placed in the incubator (at 37–38°) and the leads from these ran to the series of knife switches outside the incubator and thence to the potentiometer and electrometer. The glass apparatus was plugged with cotton-wool, sterilised in hot-air and assembled with aseptic precautions. 8 cc. of medium were placed in each cell and the inoculum was 0·1 cc. of a 16-hour culture of the well-known "Aronson" strain of haemolytic streptococci. The purity of the cultures after experiment was confirmed by film preparations and agar plate cultures. Half-hourly readings were taken for the first 12–14 hours of incubation.

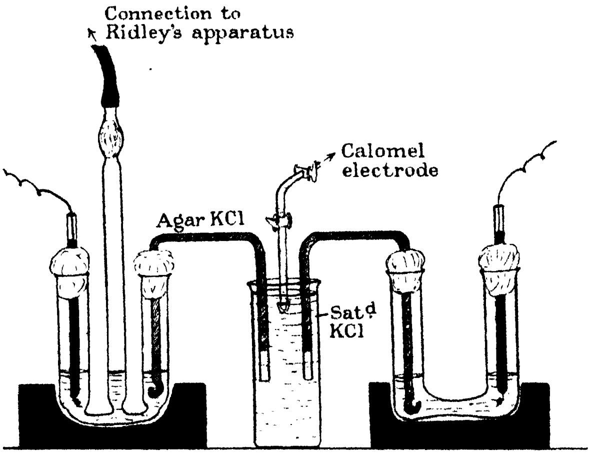

Fig. 1. Arrangement of apparatus in incubator.

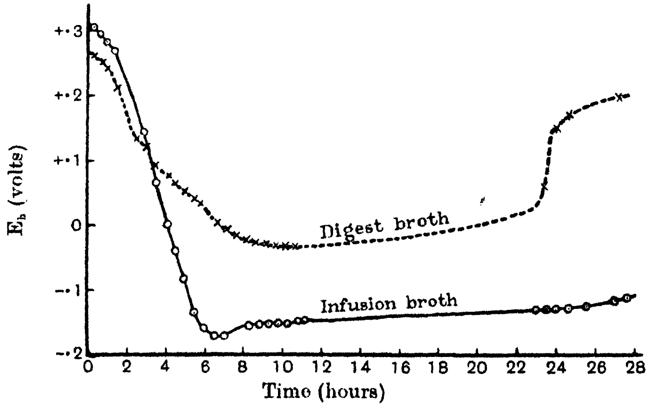

Fig. 2. Freshly boiled broths.

Results.

TRYPTIC-DIGEST AND PEPTONE-INFUSION MEDIA (FIG. 2).

Horse-flesh infusion broth, containing 2 % of Difco peptone, and Douglas's tryptic-digest broth were used. The digest broth had a slightly lower initial electrode potential, but the potential of cultures in this broth fell more slowly and did not reach as low a level as in the infusion broth. The curves suggest more opposition to reduction processes in the digest medium and there is some indication of reduction in two stages. Further differences between the two kinds of broth will become apparent in later sections.

Effect of exclusion of air (*Figs. 3 and 4*).

When access to air of an infusion broth culture is prevented by sealing with melted vaselin the potential falls rapidly at first but subsequently more slowly, reaching eventually the same value as that reached in aerobic cultures.

In sealed tryptic-digest broth cultures the potential falls as rapidly as in aerobic cultures and reaches a lower level than that of aerobic cultures in the same broth.

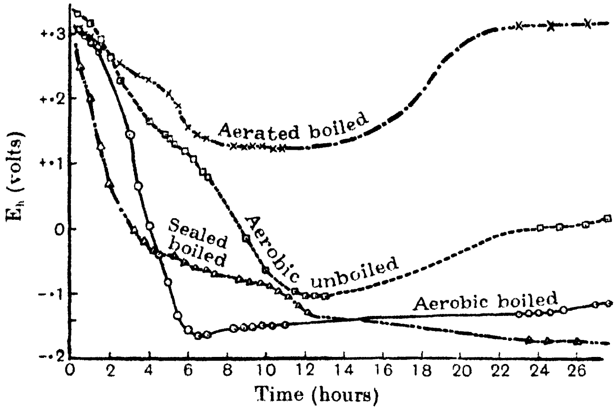

Fig. 3. Peptone-infusion broth.

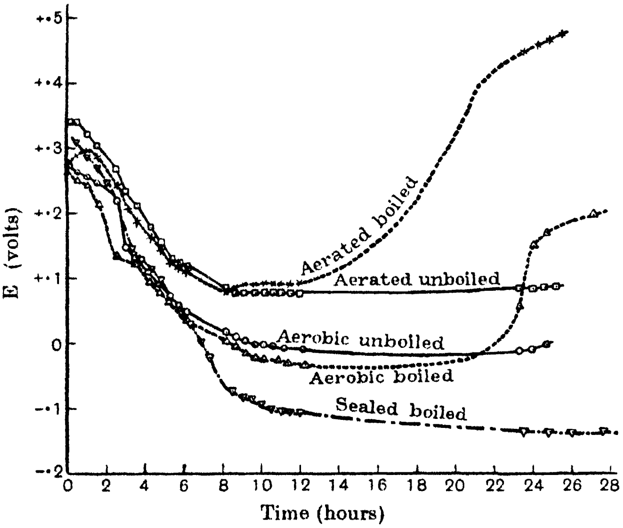

Fig. 4. Tryptic-digest broth.

Aerobically much lower potentials are reached in infusion broth but in the absence of air practically the same potentials are reached in infusion broth and tryptic-digest medium. This suggests an oxygen-carrying effect in tryptic-digest medium effected by substances liberated during tryptic digestion in the preparation of the medium. This phenomenon will be considered in more detail in a later section on the effect of serum.

Effect of freshly boiled medium (*Figs. 3 and 4*).

Tubes of broth were immersed in a boiling water-bath for 30 minutes and cooled immediately before inoculation. The electrode potential of the broth was reduced 30–100 millivolts by this treatment, and the potentials of cultures fell more rapidly and reached lower levels in freshly boiled broth. In a culture in freshly boiled infusion broth $E_h - 0{\cdot}1$ volt was reached in 5 hours, whilst 11·5 hours were required to reach this potential in untreated broth. With tryptic-digest medium, boiling had less effect on reduction processes, but the potential rose much more rapidly after the phase of active proliferation in the freshly boiled medium (see Fig. 4). This facilitation of reduction effects and acceleration of oxidising effects by boiling the medium presents analogies to the expression of water from a sponge and the subsequent rapid re-absorption.

Effect of aeration (*Figs. 3 and 4*).

Aeration was effected by Ridley's [1928] apparatus, the cultures being alternately raised and depressed in cells of the type shown on the left-hand side of Fig. 1, fresh surfaces of the culture thus being constantly exposed to the air. When aerated in this way the potentials of cultures fell very slowly and did not reach the low levels observed in stationary aerobic cultures, and, after the phase of active growth, the potentials rose rapidly, in some cases reaching values corresponding to the formation of peroxide.

In freshly boiled broth the potentials rise much more rapidly after the phase of active growth, particularly in tryptic-digest media, thus providing a further example of the "sponge effect" already mentioned.

Effect of peroxide (*Fig. 5*).

When an abundant supply of oxygen is available, haemolytic streptococci may form peroxide, and this has a marked effect on the oxidation-reduction potential. In saline 0·02 % hydrogen peroxide has $E_h + 0{\cdot}52$ volts. When peroxide was formed in aerated broth cultures (as detected by the benzidine-peroxidase test) the E_h registered between + 0·46 and + 0·5 volt according to the amount present. The upper curve in Fig. 5 shows the potentials observed in a tryptic-digest broth culture to which 0·007 % hydrogen peroxide was added before inoculation. It will be observed that there is a lag period before the potential commences to fall appreciably. After this period, when the peroxide has disappeared, the potential-time curve is almost identical with that of a plain tryptic-digest broth culture inoculated about 24 hours later than the peroxide culture. Incubation of the culture with the peroxide for some 8 hours appears not to have any appreciable effect on the organisms since the usual potential-time curve is obtained. This is in agreement with observations made on *B. sporogenes* [Quastel and Stephenson, 1926].

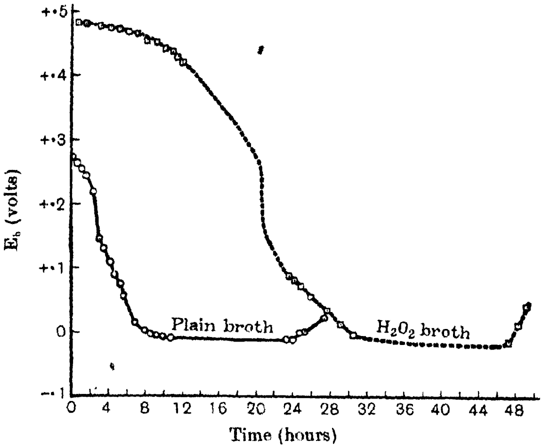

Fig. 5. Unboiled tryptic-digest broth.

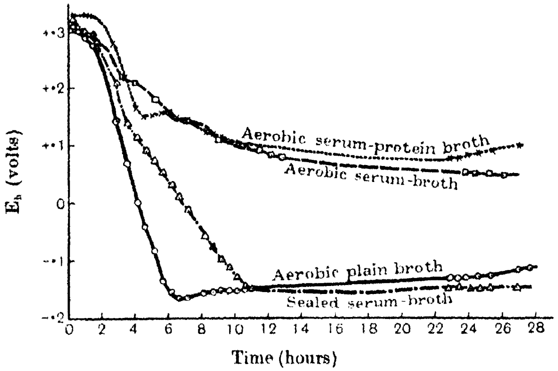

Fig. 6. Peptone-infusion broth.

Effect of serum (Fig. 6).

The presence of serum has a remarkable effect on the potentials reached by aerobic cultures of haemolytic streptococci. In aerobic 50 % serum-broth (peptone-infusion medium) the potential does not fall below E_h + 0·05 volt despite the very luxuriant growth, whilst in plain peptone-infusion broth the potential drops to below − 0·15 volt. The same effect on the potential was obtained when separated serum-proteins were used instead of native serum. Serum-proteins were prepared by three precipitations by means of sodium chloride and acetic acid, dissolving in water, dialysing to remove excess salt, adjusting the reaction and making up to the original volume of serum.

When 50 % serum-broth cultures were sealed with vaselin the potential fell to the same level as that reached in aerobic plain broth cultures (*i.e.* some

200 millivolts lower than in aerobic serum-broth cultures). Evidently, then, serum has an oxygen-carrying effect in the presence of air, and this is due to the serum-proteins. This oxygen-carrying effect may be ascribed tentatively to the sulphur-containing groups in the protein molecule. The mechanism of oxidation of a substance, BH_2, in the broth in the presence of protein, PrS.SPr may be represented as follows:

$$\mathrm{PrS.SPr} + BH_2 \rightarrow 2\mathrm{PrSH} + B$$

and in the presence of air

$$2\mathrm{PrSH} + \tfrac{1}{2}O_2 \rightarrow \mathrm{PrS.SPr} + H_2O.$$

In a sealed culture without access to atmospheric oxygen this oxygen-carrying effect could not continue—thus conforming to the experimental results.

It will be recalled that tryptic-digest broth possesses this oxygen-carrying effect to some degree and it would seem possible that, during the tryptic digestion in the preparation of the medium, sulphur-containing compounds are brought into solution, thus conferring the oxygen-carrying effect on the broth.

Effect of sulphur compounds (Figs. 7 and 9).

In view of the participation of sulphydryl compounds in biological oxidation-reduction systems [Hopkins and Dixon, 1922; Meyerhof, 1923] and the oxygen-carrying effect of serum, the influence of two compounds containing the —S.S— linking on the potentials has been studied.

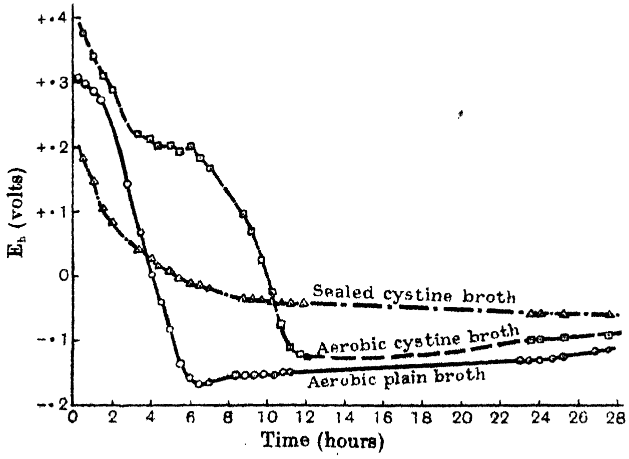

Fig. 7. Freshly boiled infusion broth.

In aerobic broth cultures containing 0·02 % (approximately) of cystine (see Fig. 7) the potential falls at first and then there is a very flat portion of the potential-time curve, after 4 to 6 hours' growth, just as with serum-proteins (Fig. 6). In sealed 0·02 % cystine-broth cultures there is a rapid initial fall of potential, but the level reached is not as low as in sealed serum-broth cultures. A distinct similarity can be traced between the effect of serum and of cystine but the differences suggest the co-existence of some other effect.

Since cystine is relatively insoluble, the influence of thiodiglycollic acid was studied (Fig. 8). In 0·05 % thiodiglycollate-broth there is again the flat portion of the curve after 4 to 6 hours' growth in aerobic cultures and the rapid initial fall in sealed cultures. In addition, in the sealed cultures a potential some 50 millivolts more negative is reached than in the aerobic cultures. We have here, therefore, direct evidence of the oxygen-carrying effect of sulphur compounds and support for the hypothesis that these may account for the influence of serum on the potentials reached in cultures. That the effects observed are not absolutely identical may be due to the different range of effect of sulphur compounds when combined in the protein molecules, or to the effect of other constituents of the protein complex, *e.g.* lipins [Lepper and Martin, 1929].

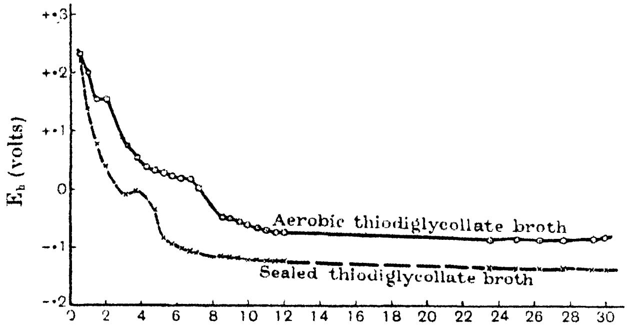

Fig. 8. Freshly boiled infusion broth.

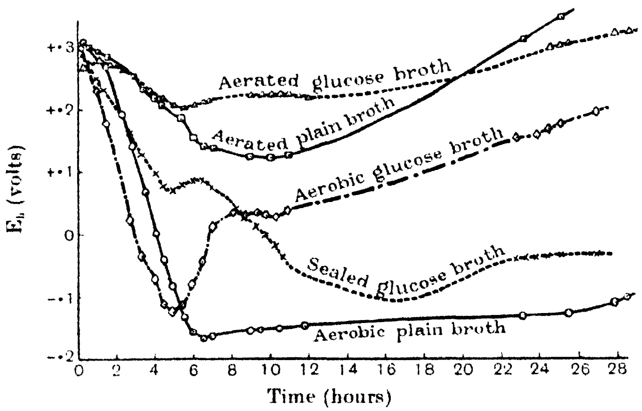

Fig. 9. Freshly boiled infusion broth.

Effect of glucose (*Fig. 9*).

In 1 % glucose-broth cultures the electrode potential falls very rapidly for about 5 hours and then suddenly rises some 100 millivolts. This is followed by a slight fall in potential and then a gradual increase. Acid is produced in glucose-broth cultures about the time of the sudden rise in potential, and although the direct effect of p_H on the electrode potential is most probably insufficient completely to account for the phenomenon, this, together with the indirect effect of the acidity on the constituents of the culture may explain the effects produced. When air is excluded by a vaselin seal the potential in glucose-broth cultures falls more slowly than in aerobic cultures. This surprising result suggests that oxygen is required for the rapid development of high reduction potentials. Since the first stage of glucose fermentation (to lactic acid, etc.) proceeds anaerobically it would seem that the oxygen is required either for the utilisation of food-stuffs in the broth, or for utilising lactic acid, etc. if these are utilised by the streptococcus. When glucose-broth cultures are aerated vigorously growth is very luxuriant but the potential falls only to a very limited extent (some 100 millivolts compared with 450 millivolts in aerobic plain broth cultures). After the cessation of active proliferation the potential rises very slowly and does not reach the high oxidation potential reached in aerated plain broth; glucose or its breakdown products apparently opposing oxidation processes. Dubos [1929] has suggested that reductive conditions are necessary for active proliferation, but the high potential maintained in aerated glucose-broth cultures is accompanied by luxuriant growth, so that the reaching of a high reducing potential does not seem essential. Also in anaerobic sealed cultures, where there is little oxygen to oppose reduction processes, the potentials indicate a slower establishment of intense reducing conditions than in aerobic cultures, again suggesting that the organisms do not favour highly reducing conditions.

Effect of intermediate fermentation products (*Figs. 10 and 11*).

The effects of 1 % of the sodium salts of lactic acid, succinic acid and fumaric acid on the potentials of broth cultures of haemolytic streptococci are shown in Figs. 10 and 11. In aerobic cultures the potential falls rapidly—most rapidly with succinate and least so with fumarate broth cultures. When sealed with vaselin the potential falls very slowly in 1 % fumarate broth, somewhat more rapidly in broth containing both 1 % fumarate and 1 % succinate and still more rapidly in 1 % fumarate-, 1 % lactate-broth. The slow fall in potential in anaerobic cultures is probably due to the lack of oxygen for the utilisation of the acids. In the case of fumarate-lactate-broth the fumaric acid may act as hydrogen acceptor [Thunberg, 1921], as in the presence of muscle, thus oxidising the lactic acid to pyruvic acid which is probably readily utilised by bacteria.

$$\underset{\text{lactic acid}}{CH_3.CH(OH).COOH} + \underset{\text{fumaric acid}}{COOH.CH:CH.COOH} \rightarrow \underset{\text{pyruvic acid}}{CH_3.CO.COOH} + \underset{\text{succinic acid.}}{COOH.CH_2.CH_2.COOH}$$

In aerated cultures the potentials have a delayed fall followed by a slow rise in potential. This retarded rise in potential, also shown in glucose-broth, is in contrast with the observation of Platt [1927] that glucose and lactic acid accelerated the production of peroxide by pneumococci.

Conclusions.

Readily reproducible potential-time curves, varying markedly with change of conditions, were obtained with cultures of haemolytic streptococci. By varying cultural conditions the electrode potential during growth may fall over 450 mv., or less than 80 mv., and, after the phase of active growth, the potential may remain at a very low level, corresponding to intense reducing conditions, or may rise rapidly to levels at which peroxide appears. These very different potentials reached indicate the very wide range of oxygen tension at which the streptococci will grow readily, and must produce very different effects. Fermentation reactions, products of metabolism, morphology, virulence and toxin production can all probably be correlated with the oxidation-reduction potentials developed in the medium.

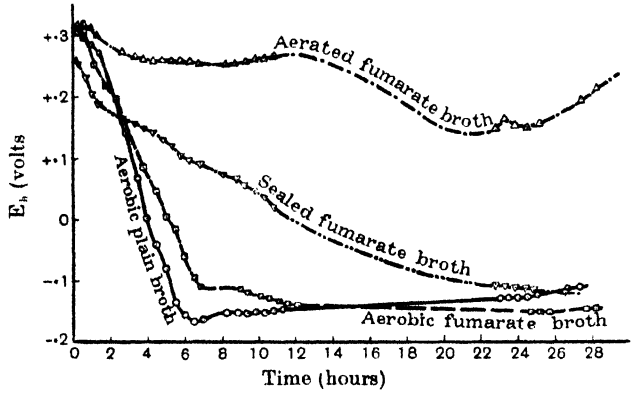

Fig. 10. Freshly boiled infusion broth.

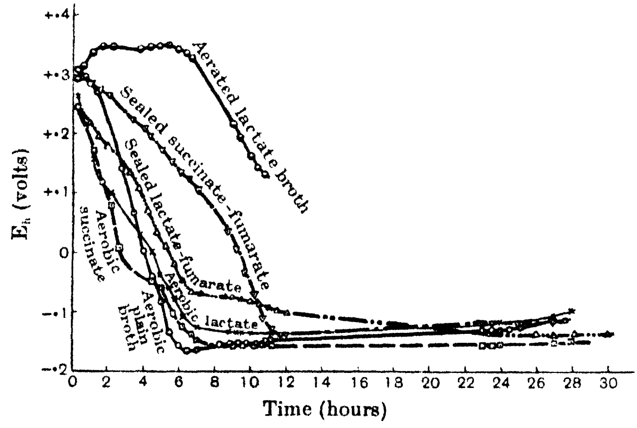

Fig. 11. Freshly boiled infusion broth.

In aerobic 50 % serum-broth cultures the potential falls only 250 mv. during growth compared with a fall of 450 mv. in plain broth cultures. This oxygen-carrying effect, which is due to the serum-proteins and occurs only in aerobic cultures, is of interest from several points of view. With regard to the mechanism of the effect, some evidence is presented that this may be due to the sulphur-containing units of the protein molecule. It seems possible that —SH groups in proteins may behave similarly to those in peptides such as glutathione. Serum-broth is a common culture medium for haemolytic streptococci and it seems probable that cultural conditions in serum-broth may approximate to those in the blood-stream. The special biological characteristics of organisms recently isolated from the body or obtained by animal passage may be due to the effect of serum-proteins, which, by regulating the oxidation-reduction potential, would control the metabolism of the streptococci.

Fildes [1929] has observed that the potential of subcutaneous tissue is too high for the germination of tetanus spores, and this again may be due to the maintenance of relatively high potentials through the agency of the oxygen-carrying effect of proteins.

The potential-time curves give some information regarding peroxide formation by haemolytic streptococci. In vigorously aerated cultures peroxide appeared after 16 to 24 hours' growth. In every culture in which peroxide was detected the electrode potential was high, *i.e.* between + 0·46 and + 0·52 volt according to the amount of peroxide formed. The same potentials were observed when hydrogen peroxide was added to saline or to broth. Bacterial peroxide is therefore closely similar to hydrogen peroxide. Low concentrations of hydrogen peroxide appeared not to have appreciable toxic effects on the streptococci but merely to produce a lag phase. In an aerobic tryptic-digest broth culture to which 0·006 % hydrogen peroxide was added the usual potential-time curve was obtained after the long lag period. In this stationary aerobic culture peroxide again appeared after 5 days' incubation. Glucose and lactic acid retarded peroxide formation whilst Platt [1927] found that with pneumococci they accelerated peroxide formation.

In describing the state of oxidation-reduction systems the electrode potential, E_h, is sometimes replaced by r_H, which, by analogy with p_H, is the logarithm of the reciprocal of the partial pressure of hydrogen in equilibrium with the system.

In the following table are summarised the minimum values of r_H reached in various cultures and the time required to reach these values.

System	Approximate minimum value of r_H	Time to reach r_H (hours)
Hydrogen peroxide in broth	30	0
Bacterial peroxide	30	0
Uninoculated broth	25	0
Aerated glucose broth cultures	21	5
Aerated plain ,,	17	8
Aerobic serum ,,	16	16
Sealed ,, ,,	9	12
Freshly boiled aerobic glucose broth cultures	10	5
,, sealed ,, ,,	10	24
,, aerobic digest	13	12
,, sealed digest	9	24
Unboiled aerobic infusion	10·5	12
Freshly boiled aerobic infusion	8·7	7
,, sealed ,,	8·7	20

Dubos [1929] claims that reductive conditions are necessary for the rapid proliferation of small implants of haemolytic streptococci, but it has been found that in the present experiments the streptococci showed particularly luxuriant growth under conditions in which the potential remained at a high (oxidising) level and that anaerobically the fall in potential in glucose, lactate, and similar broth cultures was slow.

Distinct differences exist between the potential-time curves of different strains of haemolytic streptococci. In this communication results obtained with one strain only are reported, but most of the phenomena described have been observed with several different strains.

SUMMARY.

1. Experimental details are described for obtaining reproducible oxidation-reduction potential-time curves of cultures of haemolytic streptococci. The potentials observed varied widely with different cultural conditions.

2. The presence of serum in broth inhibits the fall in potential during growth in aerobic cultures. This influence is due to an oxygen-carrying effect of the serum-proteins which has been tentatively ascribed to sulphur-containing units in the protein molecule. The biological significance of these phenomena is discussed.

3. The fall in potential during growth is less in tryptic-digest medium than in peptone-infusion broth.

4. In freshly boiled broth the potential falls more rapidly and to a lower level than in unboiled broth, and, particularly in tryptic-digest medium, rises more rapidly after the cessation of active proliferation.

5. When cultures are aerated the potential falls less during growth and rises more rapidly after the logarithmic phase of multiplication. Peroxide is frequently formed in aerated cultures and has a marked effect on the potentials, which are similar to those observed when hydrogen peroxide is added to broth or to saline. Glucose, lactic acid, etc. retard peroxide formation. When a low concentration of hydrogen peroxide is added to broth before inoculation there is a long lag period but after this the normal potential-time curve is obtained. The organisms, therefore, are apparently not injured by the peroxide in low concentration.

6. The potential falls rapidly in aerobic 1 % glucose-broth cultures but less rapidly in sealed cultures. A short phase of rapid increase of potential is observed in aerobic glucose cultures after a few hours' growth. In aerated glucose-broth cultures the potential falls very little despite the very luxuriant growth obtained.

7. No evidence was obtained that reductive conditions (low oxidation-reduction potential) favoured the growth of haemolytic streptococci.

The author is indebted to Dr R. G. White and Dr E. W. Todd for suggestions, assistance and helpful encouragement.

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LX. STUDIES IN GAS PRODUCTION BY BACTERIA.

I. APPARATUS FOR THE MEASUREMENT OF THE RATE OF GAS PRODUCTION.

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(*Received February 14th, 1930.*)

Bacterial activity is normally associated with the absorption or evolution of gases. The production of gases may be due to ordinary respiratory evolution of CO_2, or to the disintegration of nutritive compounds whereby gaseous by-products are formed. The absorption of gas is usually to be correlated with respiration where oxygen is absorbed from the atmosphere.

When bacteria cultivated in broth absorb or evolve relatively large amounts of gas, volume changes may be measured with considerable accuracy, and thus may throw some light on the metabolism of the organisms. In particular, regular observations of volume changes over a period of time should determine the activities of the bacteria in various stages of the life of the culture.

In the course of researches on bacterial denitrification, three experimental methods were devised. The results of this work are published in the succeeding paper; the present paper describes the apparatus used.

I. The pyknometer method.

The first method was devised for use in an incubator, where compactness is an advantage. A culture tube A (Fig. 1), of volume approximately 10 cc., is filled to the neck with a nitrate- or nitrite-broth and sterilised. After inoculation it is attached by means of pressure tubing, *B*, to a pyknometer, *C*, which is initially filled with pure dry mercury. The culture tube *A* has a narrow neck for convenience in attaching the tubing; milk centrifuge tubes were found to be suitable.

When the culture attacks the nitrate or nitrite, the gases evolved displace some of the mercury into a collecting tube, *D*, which is changed periodically. The displaced mercury is weighed and its volume calculated. Hence the volume of gas produced during any desired interval of time is known.

The following precautions are adopted.

(i) The pyknometer is thoroughly cleaned with hot chromic acid, and carefully dried before use in each experiment; the mercury also is washed and dried. These operations were designed to make the mercury sensitive to even slight increases of pressure due to gas evolution. In practice, it was found that the mercury was displaced spasmodically in quantities of about 0·03 cc. at a time, equivalent to about 5 drops.

(ii) The tube *A* is filled with broth to the neck, to minimise the air space above. A small production of gas thus causes a maximum increase of pressure on the mercury, and renders the apparatus more sensitive to small volume changes. A minimal air space is an advantage in processes such as denitrification where gas production is reduced or inhibited by the presence of air.

The whole apparatus is placed after assembly in an incubator automatically regulated to the desired temperature.

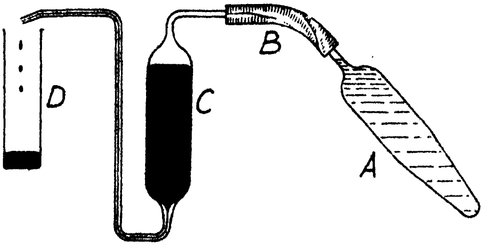

Fig. 1.

II. THE CAPILLARY TUBE METHOD.

This method was devised for making simultaneous observations on a number of cultures under the same physical conditions. The effect of varying other factors in a series of cultures could thus be determined.

The broth culture is normally contained in a centrifuge tube of about 10 cc. capacity, and connected by means of rubber pressure tubing to a glass capillary tube about 150 cm. in length. The culture tube is then completely immersed in a thermostat at the desired temperature, which should not vary during the experiment by more than 0·05°.

The capillary tube is first calibrated, thoroughly cleaned with hot chromic acid, washed, and carefully dried. A thread of mercury is then inserted to indicate gas evolution. The capillary tube is supported horizontally, lying on a paper scale by which the movement of the mercury thread can be recorded (Fig. 2). A movement of the thread of 1 cm. corresponded to an alteration in volume of 0·004–0·04 cc., according to the bore of the tube used. The total volume of gas produced by the denitrification of 25 mg. of KNO_3—about 3 cc.—could be contained in 100 cm. or less of tubing, and its production with time could be measured with an accuracy of 0·01 cc.

When it is desired to measure the rate of gas production over short intervals of time, the small tube may be replaced by a boiling-tube of about 100 cc. capacity. If desired, mechanical stirring of the broth can be accomplished by using a tube with a mercury seal [Findlay, 1923]. By filling the culture tubes as completely as possible, the influence of variation in barometric pressure is minimised, and is usually negligible. Readings of the barometric pressure were taken systematically in all experiments extending over 24 hours, but corrections are necessary only when fluctuations are great, and when more than 3 cc. of gas is produced. The influence of fluctuations of room temperature on the volume of the gas are negligible; they were found to be of the order of ½ %.

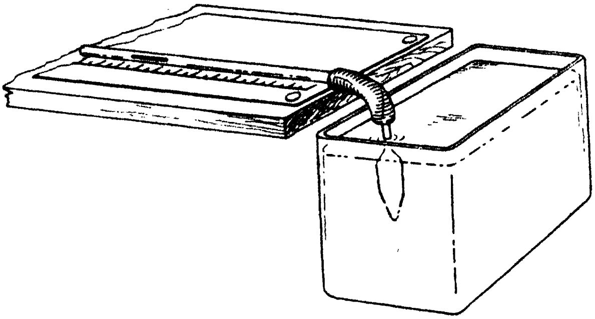

Fig. 2.

The less the volume of the air space above the culture, the more sensitive is the method. The movement of the mercury thread depends on the difference of pressure on its two ends, so that the minimum volume change sufficient to move the mercury thread is inversely proportional to the total volume of the air space.

III. LARGE-SCALE METHOD.

The third experimental procedure adopted was designed to determine the nature of the gases produced at various stages in the process of denitrification. The object therefore was to obtain quantities of gas sufficient for analysis at each of these stages rather than to obtain accuracy of measurement. However, it is possible by this method to measure the volume of gas produced in any given interval of time.

Samples of the broth can also be withdrawn periodically so that bacterial counts, p_H determinations, and chemical tests can be made.

The apparatus is shown in Fig. 3. A 5-litre flask containing the broth is fitted with a two-holed rubber stopper. Samples of the broth can be withdrawn into the gas washing bottle *C* by means of the tube *B* which extends into the middle of the flask. The gas evolved is collected in the gas-burette *D* by displacement of mercury. This vessel can be changed frequently, the screw-clip *E* being closed during the operation. The whole apparatus is placed in an incubator.

The gases so obtained can then be analysed in a Haldane apparatus.

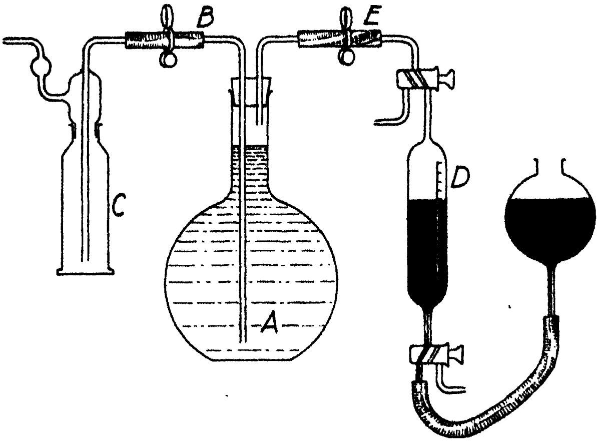

Fig. 3.

SUMMARY.

Simple methods are devised to measure the rate of production of gas continuously during the growth of a broth-culture.

REFERENCE.

Findlay (1923). Practical physical chemistry (London, 4th ed.), p. 242.

LXI. STUDIES IN GAS PRODUCTION BY BACTERIA.

II. DENITRIFICATION AND BACTERIAL GROWTH PHASES.

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(*Received February 14th, 1930.*)

THE bacterial reduction of nitrates and nitrites to free nitrogen has long been known; it has been the subject of interest in such diverse branches of science as marine biology, sewage research, and agriculture. Despite its importance, comparatively little accurate investigation appears to have been carried out: especially is there a lack of quantitative data as to the rate at which denitrification takes place.

Baur [1902] found that 5 % nitrate or nitrite in a broth culture of *B. actinopelte* was completely decomposed in 10–14 days at 15° to 20°; Drew [1911] stated that at 29° *B. calcis* destroyed 0·5 g. of KNO_3 in 86 hours. Apparently the only record of stages in the denitrification is that of Lumia [1915], who found that under favourable conditions there was after 1½ days sudden gas production at a rapidly increasing rate, and an equally rapid slowing down towards the end. He also found that with varying conditions it was the speed of the reaction that was affected and not the total denitrification expressed either in terms of the gas produced or of the amount of nitrate destroyed. Korsakova [1927], using *B. denitrificans fluorescens* in a nitrate medium, estimated periodically the quantity of nitrogen produced and the amounts of nitrate and nitrite present. The intervals between estimations were, however, too long to show the progress of the denitrification in detail.

It is apparent from the above record that little work of value has been done on the rate of denitrification. The numerous factors that affect the rate do not seem to have been appreciated; otherwise the isolated items of information given would have been recognised as haphazard. With the exception of Carapelle [1908], who stated that the activity of bacteria possessing reducing powers is more marked in young than in old cultures, no one seems to have correlated the rate of reduction with the age of the culture. Still less has it been realised that in denitrification the rate of gas production may under certain circumstances be taken as a measure of the number of viable organisms present; or that there is a delay in gas production which can be correlated with the lag phase of bacterial growth, and which furnishes a simple method of studying quantitatively the factors affecting the duration of the lag phase. The rate of denitrification is greater in a young culture than in an old one for two reasons: (i) the number of bacterial cells is greater in a young culture than in a senescent one; (ii) the majority of cells in a young culture are in a state of active cell-division, so that each attacks the nitrate more rapidly than a cell from an older culture.

The utility of denitrification to the organism is primarily a respiratory one, and therefore the rate of denitrification, the rate of respiration, and the rate of bacterial growth are interdependent; and a curve showing the production of nitrogen with time for a given organism under given conditions should be capable of correlation with the growth curve of the culture.

In the preceding paper [Cranston, 1930], methods are described for measuring the rate of gas production by bacteria when cultivated in a liquid medium. For the following work, the device found most convenient and simple to use was that of attaching a 10 cc. culture tube with a narrow neck to a capillary tube containing a thread of mercury; it was employed in the work described in this paper, which is the outcome of studying the rate of denitrification of over 90 cultures.

General form of the denitrification curve.

When the volume of the gas absorbed or produced during denitrification is plotted against the time from seeding, a curve of the general type shown in Fig. 1 is obtained; such a curve will be referred to throughout this paper as a "denitrification curve." The rate of change in volume varies in magnitude and sign so that as many as nine stages can be differentiated. From this curve another may be obtained by plotting the increments of volume in selected intervals of time against the mean time from inoculation. Except for the initial contraction *OC* and the final contraction *JK*, the curve will represent the rate of gas evolution at various stages in the growth of the culture; such a curve will be referred to throughout this paper as a "rate of denitrification curve."

The initial contraction indicated by the portion *OC* of the curve is due to the absorption of atmospheric oxygen present in the air space between the surface of the medium and the mercury thread in the capillary tube; it is effected partly by the oxidation of the medium and partly by the respiration of the bacteria. The control experiments discussed later show that the contraction in this phase along *OA* is due almost entirely to absorption by the medium, while the portion *AC* is due partly to aerobic bacterial respiration. The final contraction *JK*, which when it occurs is usually of very small magnitude, can be attributed to the same causes.

The rate of denitrification curve (Fig. 2, p. 534) shows general similarity to a bacterial growth curve, and this resemblance suggests that the two may be correlated. A growth curve can be dissected to represent various phases of bacterial growth: it will be appropriate to discuss these before dealing with the interpretation of the rate of denitrification curve.

Growth phases in bacterial cultures.

The rate of growth of bacteria in a sub-culture varies greatly with time [Buchanan and Fulmer, 1928]. It has been established that in general there is no appreciable initial increase in the numbers of bacteria [Lane-Claypon, 1909; Penfold, 1914; Ledingham and Penfold, 1914]. After germination has begun, cell-division proceeds slowly at first and then more and more rapidly until such time as the bacteria are dividing at a uniform rate, *i.e.* until they are increasing in number geometrically over equal intervals of time. The rate of increase then diminishes progressively until the numbers of bacteria remain constant, after which there is a diminution in numbers until the culture dies off. Buchanan differentiates seven such phases as follows.

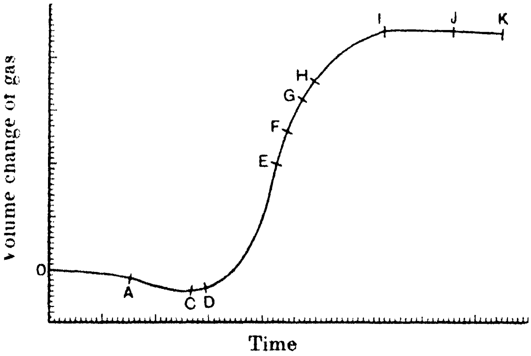

Fig. 1. General form of a denitrification curve, marked off into sections which are correlated with bacterial growth phases.

1. *Initial stationary phase.* During this phase the number of bacteria remains constant. The duration of the phase varies with the experimental conditions, and with the organism used.

2. *Phase of positive growth acceleration.* Here the cells are beginning to divide, and the average rate of increase in numbers increases with time. This phase extends over a period of time because all the cells do not germinate at once. When all the viable cells of the original inoculum have germinated they continue to divide regularly, and the rate of increase is then constant; this gives rise to the third phase.

The term "lag phase" has been defined as covering the period which elapses between "the time of seeding and the time at which maximum rate of growth begins" [Chesney, 1916]. Buchanan, however, in a desire to differentiate between these two phases has suggested that the term should be reserved specifically to indicate the second phase, viz. "that period elapsing between the beginning of multiplication and the beginning of the maximum rate of increase per organism." It appears to us unfortunate that he did not suggest the use of the term for the first phase instead of the second. The word "lag" is more suggestive of the first phase, and indeed in discussions of theories of the lag phase, it has been so frequently used by various workers, including Buchanan himself, to mean the stationary phase that this meaning has been sanctioned by custom.

3. *Logarithmic growth phase.* During this phase the rate of increase per organism remains constant.

4. *Phase of negative growth acceleration.* Here the rate of increase decreases, although the number of bacteria increases.

5. *Maximum stationary phase.* In this phase the number of bacteria remains constant.

6. *Phase of accelerated death.* During this phase the number of bacteria is decreasing, slowly at first and then with increasing rapidity.

7. *Logarithmic death phase.* Here the rate of decrease per organism remains constant. Buchanan and Fulmer [1928] state that such decrease is seldom logarithmic.

Comparison of growth phases with phases of gas evolution.

It is generally agreed that the significance of bacterial denitrification is a respiratory one, *i.e.* the oxygen available from the nitrate may be supplementary to, or may be a substitute for, free atmospheric oxygen. Most writers find that the presence of free oxygen retards denitrification [Kunnemann, 1898; Caron, 1912] and in some cases may even inhibit it completely [Gran, 1901; Ampola, 1907]. It does not seem to be clearly recognised, except perhaps by Korsakova [1927], that the reduction of nitrate to nitrogen occurs by stages with the formation of intermediate compounds, and that gas is produced only in the last stage. Denitrification is therefore an indication of the completeness with which oxygen is utilised, and this in turn depends on the amount of nitrate present. Statements regarding inhibiting factors should therefore only be made when the concentration of the nitrate is taken into account. This point is discussed in detail later in the paper when experimental data enable it to be done quantitatively.

The denitrifying consequence of bacterial respiration should lead to the establishment of some connection between the rate of gas evolution and the number of viable organisms present: but if that relationship is to be made quantitative, the following conditions must be satisfied.

1. The organism must depend solely upon the nitrate or nitrite for its oxygen supply.

2. The gas evolved is pure nitrogen.

3. All the bacteria consume oxygen at the same rate, this rate being independent of the age of the individual organism.

4. No time lag must occur between the absorption of oxygen and the production of nitrogen.

Condition 1 can be controlled experimentally, and Condition 2 can be arranged by selecting an organism known to produce nitrogen only. No evidence is available regarding Condition 3. Even if the rate of consumption of oxygen is not proportional to the number of bacteria present, it is at least a measure of their aggregate metabolic activity, which is perhaps as significant. Condition 4 does not hold throughout the whole course of denitrification. Korsakova [1927] found by analyses performed during the course of denitrification that the nitrogen could not be completely accounted for as nitrate, nitrite, and free nitrogen, and therefore concluded that some of it existed as an intermediate compound. Ampola and Ulpiani [1899] suggested that hyponitrite is formed as an intermediate product in denitrification, but were unable to demonstrate its existence during gas production. The authors show later on in this paper that their results can be quantitatively accounted for on the assumption that this intermediate compound is the hyponitrite. Thus the reduction of potassium nitrite on this supposition would occur in two stages.

$$2KNO_2 - 2O = K_2N_2O_2$$
$$K_2N_2O_2 - O = K_2O + N_2.$$

A large proportion of the oxygen may thus be utilised before nitrogen is evolved at all. Under the conditions of the authors' experiments, these reactions do not occur simultaneously, but the nitrogen is given off only after the nitrite has been completely converted into an intermediate compound.

The number of bacteria present in a nitrite-broth culture is therefore proportional to the rate of gas evolution only through part of the life of the culture, viz. soon after gas evolution has begun. It is characteristic of denitrification curves that soon after gas is evolved, it is produced at its maximum rate. This seems to show that gas is not evolved till the later stages of the phase of logarithmic growth. The authors propose later to find if potassium hyponitrite can be used instead of potassium nitrite as a source of oxygen for the bacteria; the amount of nitrogen evolved would then be proportional throughout to the amount of oxygen consumed, and thus the earlier growth phases could be studied directly.

The growth phases detailed above may best be compared with the phases of gas evolution by considering the rate of denitrification curve given in Fig. 2.

The initial stationary phase is represented by *AC*. Experiments to be described later show some of the factors which determine its duration.

The phase of positive growth acceleration is represented by *CD*. As explained above, the production of nitrogen from nitrite is delayed through the formation of an intermediate compound; this occurs during the present phase so that the initial growth of bacteria is not accompanied by gas evolution. Ordinarily, therefore, the growth during this phase cannot be studied by gas production.

The logarithmic growth phase is represented by *DE*. In most of the experiments gas is first evolved during this phase, but whether it occurs at the early or the late part of the phase cannot be determined from the data of gas evolution alone.

The phase of negative growth acceleration, the maximum stationary phase, and *the phase of accelerated death*, represented by *EF*, *FG* and *GH* respectively, are usually of short duration. An investigation of these phases would require readings taken at intervals not greater than half an hour, and to attain any accuracy the volume changes would have to be magnified by using not less than 100 cc. of broth culture.

The logarithmic death phase is represented by *HI*. In most cases the numbers of bacteria were found to decrease logarithmically over measurable periods of time. In one experiment this period extended over 5 days [Cranston and Lloyd, 1930].

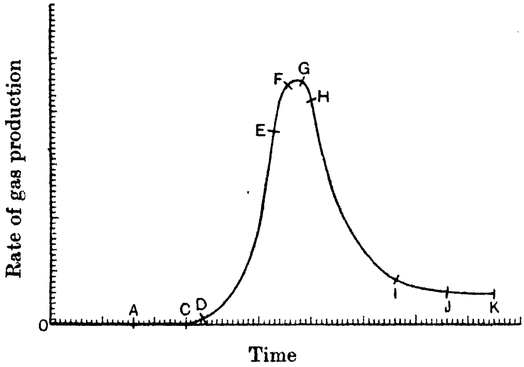

Fig. 2. General form of a rate of denitrification curve, marked off into sections which are correlated with bacterial growth phases.

The phase of slow decline, represented by *IJ*, is shown by nearly all cultures. Some experiments that throw light on these phases are described below.

EXPERIMENTAL.

For the following work, an actively denitrifying marine organism was selected. The strain employed was isolated from Loch Striven in the Clyde Sea Area at a depth of 20 fathoms. The organism will be described in detail in a forthcoming paper; a culture has been lodged with the National Collection of Type Cultures at the Lister Institute. The following are its principal characteristics.

Designation. Laboratory organism XIV.

Morphology. Vibrio, single, sometimes forming short chains, gram-negative, actively motile, length about 1·5μ.

Agar cultures. Slope cultures, thin coherent wrinkled films; surface colonies on agar plates up to 5 mm. in diameter, with wrinkles often radially arranged.

Gelatin cultures. No liquefaction.

Broth cultures. Uniform turbidity, with coherent wrinkled surface pellicle, the nature of the pellicle varying with the temperature of incubation; surface films absent from a young nitrate or nitrite broth.

Optimum temperature. 35°.

Size of the inoculum.

The general procedure adopted in making an inoculation was designed to make the seeding as constant in amount as possible. In order to obtain some estimate of the number of bacteria used in an inoculation, the direct method of counting by means of a Thoma haemacytometer was employed. One loopful of a broth culture actively producing gas was transferred to approximately 10 cc. of the sub-culture. In the parent culture the average number of bacteria counted in unit volume, $1/20 \times 1/20 \times 1/10$ mm.^{3}, was 40, which is equivalent to 160×10^6 per cc. In the sub-culture, only one bacterium was seen in 40 unit volumes examined, equivalent to 10^5 per cc. The dilution performed by transferring one loopful to 10 cc. was thus approximately 1 in 1600. This figure was checked by ascertaining the average weight of a loopful of broth to be 5 mg., which in 10 cc. gives a dilution of 1 in 2000.

The seeding was thus of the order of 1,000,000 bacteria.

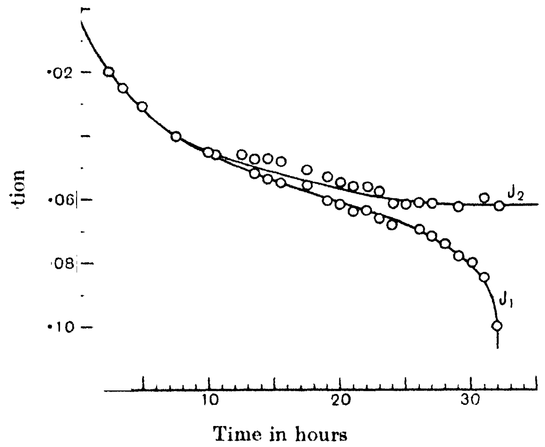

Fig. 3. Curves showing absorption of oxygen by a broth culture $J1$, and a sterile control $J2$.

A. *The initial contraction phase.*

The marked effect of aeration on denitrification has already been alluded to. It may be studied quantitatively by determining the factors that give rise to the initial contraction phase.

(*a*) *In absence of nitrates and nitrites.* When a culture tube of about 10 cc. capacity is filled with a broth medium and connected up to a capillary tube as explained in method 2 of the previous paper, there is an air space of about 1 cc. between the surface of the broth and the end of the mercury thread in the capillary tube. Unless oxygen is excluded from this space, there is a slow contraction in volume due to oxidation of the medium. Some actual experimental results are shown in Fig. 3. $J2$ was a nitrite-free sterile broth control, in which the rate of contraction slowed down as the surface of the broth became oxidised. $J1$ was a nitrite-free culture, so that after the initial lag phase was over, the demand for oxygen for bacterial respiration suddenly increased; this circumstance accounts for the sudden drop in the curve. This is verified in curve *L*3 (Fig. 5), where effects were intentionally magnified by having an air space of about 5 cc. above the broth.

The initial rate of the contraction has been found to be approximately proportional to the surface of the broth exposed. For sterile broths, the rate diminishes with time, unless the connection to the capillary tube is opened and a fresh supply of air admitted. In this case the rate of contraction first increases and then diminishes slowly. The total amount of the contraction depends of course on the amount of oxygen available above the broth. In an open tube the absorption continues until the broth is completely oxidised; in Giltay's medium a dark brown colour is then produced.

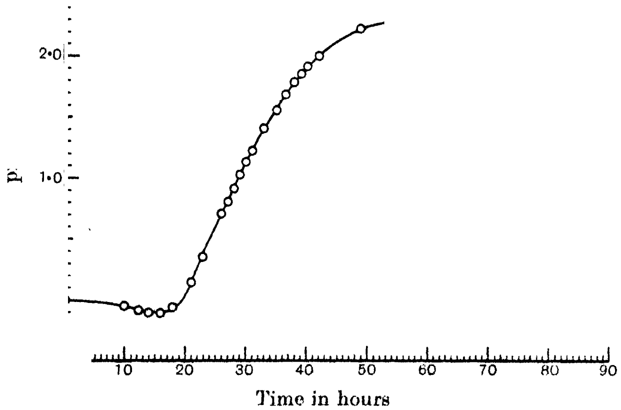

Fig. 4. A typical denitrification curve.

(*b*) *In presence of nitrites.* When a sub-culture is freshly made into a nitrite broth, the initial rate of contraction is similar to that of inoculated controls; apparently the oxygen demands of the bacteria are very small in the early part of the lag phase, and are met by the atmospheric and dissolved oxygen, and by oxygen obtained in the first stage of the reduction of the nitrite. Only when the bacteria reach the phase of rapid growth does the oxygen from these sources become suddenly inadequate, and the intermediate compound is attacked. It is for this reason that the denitrification curve of a healthy culture shows such a sharp point of inflection at the beginning of gas evolution; and attains the maximum rate of gas evolution so rapidly. Fig. 4 shows such a curve obtained from an 8 cc. broth culture (*E*2) with 25 mg. of KNO_2 added.

If a large supply of atmospheric oxygen is available, nitrate may hardly be attacked at all (*L*3, Fig. 5). Experiment shows, however, that some nitrogen is evolved even when a broth culture is spread out in a thin layer to expose a large surface for the absorption of oxygen.

(c) *Prevention of the initial contraction.* When the air above the broth and in the capillary tube was displaced by coal gas, the contraction took place in both sterile and inoculated broths, and was apparently due to the solution of some of the gases. No growth started in the inoculated broth, probably because of the toxic effect of the dissolved gases.

The effect of replacing air by nitrogen was next tried. 8 cc. of Giltay's medium containing 0·25 % KNO_3 were placed in tubes of 12 cc. capacity, thus giving a large air space to magnify any effects when atmospheric oxygen was not excluded. Six broth tubes were observed under the following conditions.

Designation	Inoculated	Nitrate mg.	Nitrite mg.	Atmosphere
*L*1	0	20	25	Air
*L*2	0	20	25	Nitrogen
*L*3	+	20	0	Air
*L*4	+	20	0	Nitrogen
*L*5	+	20	25	Air
*L*6	+	20	25	Nitrogen

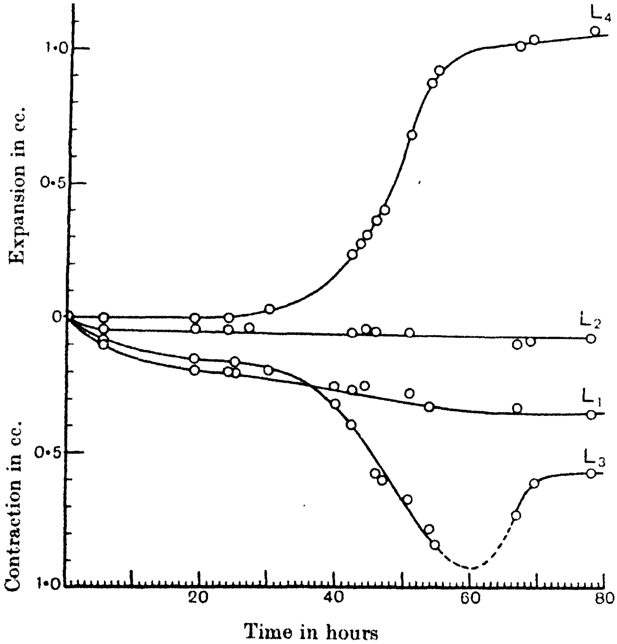

Fig. 5. Curves illustrating the suppression of the initial contraction in cultures and sterile controls on replacing air by nitrogen.

Thus *L*1 served as a control to *L*2 to determine the effect of atmospheric oxygen on sterile broth, *L*3 as a control to *L*4 to determine the effect of atmospheric oxygen on a culture, and *L*1 as a control to *L*3 to determine the contraction in the initial stages due to bacterial respiration. The denitrification curves of *L*1, *L*2, *L*3 and *L*4 are shown in Fig. 5.

No gas evolution occurred in $L5$ and $L6$, apparently because the oxygen requirements of the bacteria were satisfied by the initial stages of the reduction of the nitrate and nitrite present. In $L2$ and $L4$, which were in a nitrogen atmosphere, there was practically no contraction; but in $L1$ and $L3$, which had air, there were large contractions. On comparing the sterile control $L1$ with the nitrite-free culture $L3$, it is seen that both contracted in a similar way for the first 25 hours; the bacterial lag phase in $L3$ was then over, and a period of rapid contraction set in for the next 25 hours until the nitrate was attacked with evolution of gas, the small expansion being due to the fact that the oxygen requirements had already been partly satisfied by the oxygen of the atmosphere. $L4$, on the other hand, experienced no contraction, but at the end of 25 hours showed the expansion due to bacterial decomposition of the nitrate.

To sum up, it is found that in the absence of free oxygen there is no initial contraction; in the presence of oxygen there is an initial contraction, of a two-fold nature:

(*a*) a consumption of oxygen due to oxidation of the medium, which slows down with time;

(*b*) a consumption of oxygen due to bacterial respiration; this increases with time up to the limits of the available oxygen, but the contraction is counterbalanced by evolution of nitrogen if nitrite and nitrate are present as sources of oxygen.

B. *Factors affecting the duration of the lag phase.*

(*a*) *Effect of time of addition of nitrite.* Six cultures, $H1$–$H6$, were inoculated at the same time, and incubated at 27°. 25 mg. of KNO_2 were added immediately to $H1$ and $H2$, after a lapse of 12 hours to $H3$ and $H4$, and after 24 hours to $H5$ and $H6$. Gas production began almost simultaneously in $H1$, $H2$, $H3$ and $H4$ about 20 hours after inoculation, and in $H5$ and $H6$ about 8 hours later, *i.e.* about 4 hours after addition of the KNO_2. The following inferences were drawn.

(i) The time that elapses between the inoculation of a culture into a nitrite broth and the evolution of gas is due mainly to the lag in the development of a healthy culture. When, as in the above experiment, the period of this lag is about 20 hours, the time of first gas evolution is independent of whether the nitrite is present at the beginning or whether it is added up to 12 hours after inoculation.

(ii) The addition of nitrite to a 24-hour culture (*i.e.* one that would have begun to produce gas if nitrite had been present at the time of inoculation) does not cause immediate gas production, but there is a further delay of 4 or 5 hours. This may be due to a necessary accommodation on the part of the bacteria to its slightly altered nitrite environment, or it may more probably be due to the nitrite-reduction taking place in two stages with the formation of an intermediate compound. The first stage does not produce nitrogen.

The experiment was repeated under slightly different conditions. Six similar cultures were prepared and inoculated at the same time. They were kept at room temperature for 2½ hours, and then placed in a thermostat at 32°, and the production of gas for 100 hours measured at intervals by the capillary tube method.

KNO_2 was added to one of the cultures 2½ hours after inoculation, and to the others at intervals as shown in Table I. The last column shows the time interval from inoculation when nitrogen was first evolved, and Fig. 6 gives the denitrification curves of the series.

Table I.

Designation of culture	Time in hours after inoculation	
	KNO_2 added	Gas first produced
*HH*1	2½	13
*HH*2	5½	13
*HH*3	8½	13
*HH*4	11½	16
*HH*5	14½	19
*HH*6	18½	23

The curve for *HH*3 was spoiled because the rubber tubing joining the culture tube to the capillary tube was defective. The production of gas appeared to be as great as in the other cultures.

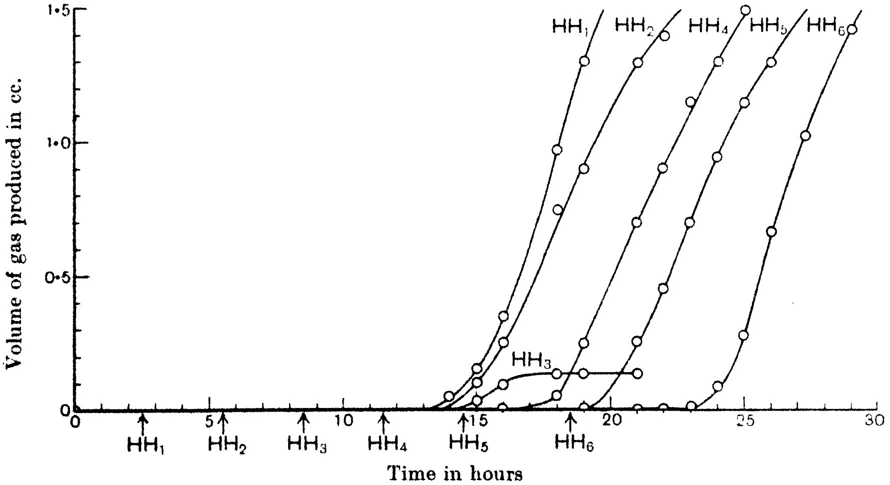

Fig. 6. Curves illustrating the effect of the time of addition of nitrite on the duration of the lag in gas production. The arrows indicate the time of addition of the nitrite to the culture designated.

This experiment confirms the previous *H* series. The same inferences may be drawn, but may be made more precise. *HH*5 and *HH*6 showed very definite growth before the addition of KNO_2; yet there was a lag of 4½ hours in each case between the addition of the KNO_2 and the rapid evolution of gas. This lag must be attributed to the formation of an intermediate compound. It is very remarkable that this same period of lag occurred in *HH* 3 and *HH* 4. The conclusion can be drawn that in the nitrite-broth culture the normal lag of 13 hours between the time of inoculation and the appearance of gas was made up of a bacterial lag of 8½ hours, and a "chemical lag" of 4½ hours.

(*b*) *Concentration of nitrite or nitrate added.* A series of similar cultures was prepared in equal amounts—about 9 cc.—of Difco broth which was adjusted before sterilisation to a p_H value of 8·5. Varying amounts of KNO_3 were added, and denitrification curves obtained at 32° (Fig. 7). The air above the broth was displaced by nitrogen so that the only source of oxygen was the nitrate.

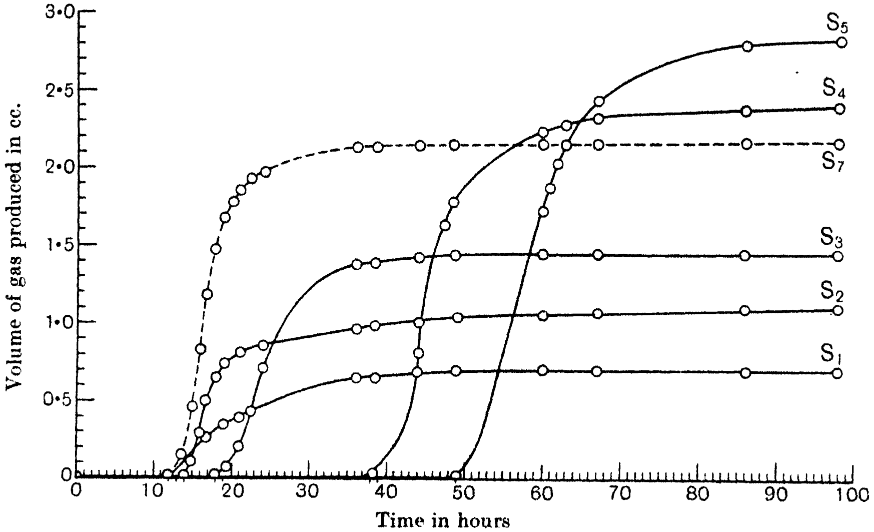

Fig. 7. Curves showing the progressive increase of the lag in gas production with increase in initial concentration of nitrate. The broken curve illustrates the difference when nitrite is used.

Table II shows that the time elapsing before evolution of nitrogen increases with the increase in concentration of the nitrate added. The experimental facts are accounted for by assuming the duration of the lag phase of bacterial growth to be about 8 hours in each culture. The numbers of bacteria then increase rapidly, satisfying their oxygen requirements by reducing the nitrate to nitrite, and then possibly to hyponitrite. No nitrogen is produced until these intermediate reductions have been completed or nearly completed, which first occurs of course in the culture with least initial amount of nitrate. The other cultures begin to evolve nitrogen in the sequence of their nitrate concentrations.

At the same time as these nitrate-broth cultures were experimented with, three nitrite-broth cultures were observed under identical conditions in order to test the validity of the following hypotheses. If nitrate is reduced in three stages, viz. to nitrite, to hyponitrite, and to nitrogen, and these stages do not occur simultaneously to any great extent so that nitrogen is not produced until the first two stages are nearly over, it was expected that (i) a nitrite-broth culture would show a considerably shorter lag in gas production than a culture containing an equivalent amount of nitrate. If hyponitrite is the intermediate compound formed, and if it is not attacked until all the nitrate and the nitrite is reduced, it was expected that (ii) a nitrite-broth culture would show the same duration of lag in gas production as a culture in a broth containing half the equivalent amount of nitrate, because as is shown in equations for *S* 4 and *S* 7 below, twice as much oxygen must be taken from nitrate as from an equivalent amount of nitrite to produce the hyponitrite.

The curve *S* 7 was obtained from one of three cultures, *S* 6, *S* 7 and *S* 8, which differed from the others in that 20 mg. of potassium nitrite were added and no nitrate. In each of these cultures, 13 hours elapsed before nitrogen began to be evolved. 20 mg. of KNO_2 are equivalent molecularly to 24 mg. of KNO_3, and it will be noted that the quantity of nitrogen produced by *S* 7 is only a little less than that produced by *S* 4 which contained 24 mg. of KNO_3. Whereas, however, *S* 7 produced nitrogen 13 hours after inoculation, *S* 4 did not do so until 37 hours had elapsed. The culture *S* 2, which contained 12 mg. of KNO_3, and which therefore required the same oxygen consumption to convert the nitrate into hyponitrite as is required in *S* 7 to convert the nitrite into hyponitrite, first produced nitrogen 13 hours after inoculation, *i.e.* simultaneously with *S* 7.

Thus the two expectations were realised, and quantitative evidence is produced to show that hyponitrite is formed during nitrate-reduction, and that, under the conditions of the above experiments at any rate, no nitrogen is evolved until all the nitrate and nitrite is converted into hyponitrite. The following equations therefore represent the reductions for the two cultures, *S* 4 and *S* 7, before nitrogen is evolved, if hyponitrite be assumed to be the product.

$$S4 \ldots 2KNO_3 - 2O_2 = K_2N_2O_2$$
$$S7 \ldots 2KNO_2 - O_2 = K_2N_2O_2.$$

Twice as much oxygen was consumed in *S* 4 as is *S* 7 before nitrogen was evolved, and the delay in the production of gas in *S* 4 is explained.

The extent of denitrification. Calculations were made to determine what percentage of the total available oxygen of the nitrate was utilised. It is reasonable to suppose that if the concentration of nitrate is very high originally, oxygen will not be a limiting factor in the growth of the culture. Some other factor may operate to stop the growth before the stage in nitrate-reduction that results in liberation of nitrogen is reached. Examples of these may be seen in the cultures *L* 5 and *L* 6, and to a lesser degree in *L* 3, mentioned previously. In the equation for the complete denitrification,

$$4KNO_3 - 5O_2 = 2K_2O + 2N_2,$$

it is seen that 2·5 molecules of oxygen are consumed for each molecule of nitrogen evolved. If the reaction does not go to completion, it would be incorrect to base the oxygen consumption on the nitrogen produced without allowing for the fact that the reaction occurs in stages. This allowance can be made by noting that four of these molecules of oxygen are consumed before nitrogen is evolved at all, thus

$$4KNO_3 - 4O_2 = 2K_2N_2O_2.$$

The ratio of the amount of nitrogen evolved to the total quantity in the original nitrate determines what fraction of the remaining 20 % of oxygen is consumed, because two molecules of nitrogen are evolved in this final stage for each molecule of oxygen consumed, thus

$$2K_2N_2O_2 - O_2 = 2K_2O + 2N_2.$$

The ratio of the oxygen consumed to the total amount available in the nitrate may be stated algebraically in terms of the amount of nitrogen evolved as follows. If the calculated total amount of nitrogen in a given weight of potassium nitrate is x cc., and the amount produced is y cc., then the ratio of the amount of nitrogen evolved to the amount in the salt is y/x, but the proportion of the oxygen consumed to the total amount available is

$$(2x + 0{\cdot}5y)/2{\cdot}5x.$$

Table II shows that in each culture the percentage of the available oxygen consumed, calculated from the volume of nitrogen evolved, was practically 100.

Table II.

Designation of culture	*S*1	*S*2	*S*3	*S*4	*S*5	*S*7
Wt of KNO_3 in mg.	6	12	18	24	30	20 (KNO_2)
Time in hours before nitrogen evolved	12	13	18	37	48	13
Available nitrogen, x (calculated)*	0·7	1·4	2·1	2·8	3·5	2·8
Vol. of nitrogen evolved, y	0·71	1·10	1·47	2·39	2·80	2·20
†Available oxygen (calculated)	1·75	3·5	5·25	7·0	8·75	4·2
‡Calculated vol. of oxygen consumed before nitrogen evolved	1·4	2·8	4·2	5·6	7·0	2·8
§Total oxygen consumed	1·75	3·35	4·93	6·8	8·4	3·9
% of available oxygen consumed	100	96	95	97	96	93

* All vols. of O_2 and N_2 in cc.

† This is the calculated volume at 17° and 760 mm. pressure of five-sixths of the oxygen in the nitrate, or of three-quarters of the oxygen of the nitrite.

‡ Equal to the volume of the nitrogen available in the case of the nitrite, and equal to double this volume for the nitrates.

§ $2x + 0{\cdot}5y$ in the case of the nitrates; $x + 0{\cdot}5y$ in the case of the nitrite.

(c) *Effect of the initial acidity of the broth.* Four cultures, *G*1, *G*2, *G*3 and *G*4, were made in nitrite broth buffered with mixtures of phosphate and sodium hydroxide solutions to give initial p_H values of 8·2, 8·0, 6·8 and 6·4 respectively. The thermostat was kept at 27°, and observations of gas production made regularly over a period of 575 hours. Fig. 8 shows the denitrification curves, and Fig. 9 shows the rate of denitrification curves for these cultures.

The final p_H values after 575 hours were 9·3, 9·2, 8·7 and 8·2 respectively. The phosphate solutions employed as buffers were thus unable to prevent the development of alkalinity which always accompanies denitrification. Qualitative tests for nitrites (Griess-Ilosvay) showed a trace present in $G3$, but gave negative results for the others.

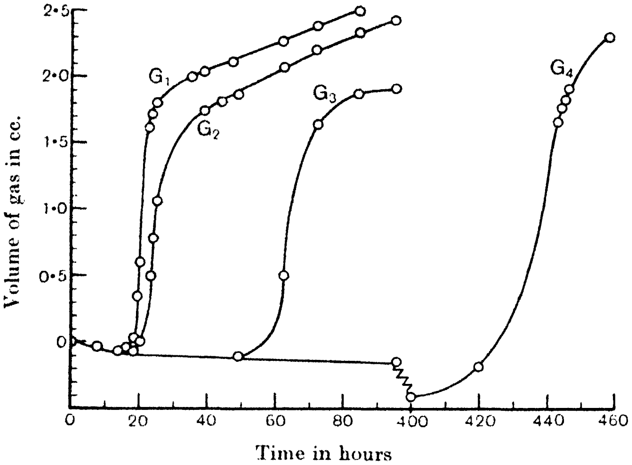

Fig. 8. Curves showing the progressive increase of the lag in gas production with increase in initial acidity of the broth. The time scale is abbreviated by omitting the interval from 96 to 400 hours.

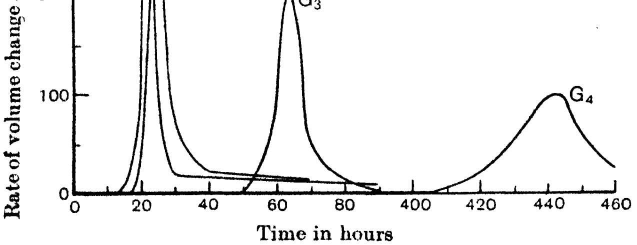

Fig. 9. The rate of denitrification curves of the G series of cultures calculated from experimental readings, some of which are shown in Fig. 8. The time scale is abbreviated as in Fig. 8.

The amount of gas produced was 2·8, 2·6, 2·1 and 2·8 cc. respectively. A rough analysis of the gas in the capillary tube at the end of 575 hours was made in each case by passing the gas several times over anhydrous $CaCl_2$

and NaOH. Less than 0·1 cc. was absorbed in each case, indicating the absence of CO_2 in amount exceeding 3 %; this agrees with more accurate analyses [Cranston and Lloyd, 1930] which showed that the gas evolved from this organism is pure nitrogen.

It is seen from the curves that increased acidity causes

(1) increase in the lag in nitrogen production, viz., 14, 18, 51 and 400 hours respectively;

(2) diminution of the rate of evolution of nitrogen when once started; this implies a corresponding diminution in the rate of increase of bacteria;

(3) progressive decrease in the maximum rate of gas production (shown by the peaks of the curves in Fig. 9), which may be taken to indicate that the maximum number of bacteria alive in the medium at any given time decreases with increased acidity.

(*d*) *Effect of temperature.* A series of similar nitrite-broth cultures, *T* 1–*T* 6, was prepared, and the six cultures were incubated at the different temperatures, 18°, 25°, 30°, 35°, 40° and 45° respectively.

A thermostat was regulated to maintain a temperature of 40°, and into this were immersed up to their necks three thermos flasks containing water at 45°, 35° and 30° respectively. The temperature of the interior of a thermos flask was so little different from that of its environment that it remained sufficiently constant throughout the experiment. Thus these three thermos flasks and the thermostat provided constant temperature baths for four of the cultures. A second thermostat, regulated at 25°, provided for a fifth culture, and the sixth was kept at room temperature in the vicinity of the first thermostat where the temperature remained constant at 18°.

The denitrification curves, which are not reproduced here, gave a series similar to those of Fig. 6, and enabled the duration of the lag phase to be determined. The results, which were confirmed by being carried out in duplicate, were as follows.

Designation of culture	*T* 1	*T* 2	*T* 3	*T* 4	*T* 5	*T* 6
Temperature, ° C.	18	25	30	35	40	45
Time in hours when gas was first produced	26	20	17	13	25	None in 50 hours

The optimum temperature for the growth of this organism under the given experimental conditions was thus about 35°. The following were found to be the two most marked effects as this temperature was departed from in either direction.

(i) A marked increase occurred in the lag phase. This was specially shown at unfavourably high temperatures, for at 45° no gas was produced in 50 hours. Incidentally, several days after the conclusion of the experiment, during which time the thermostat had been at room temperature, the culture *T* 6 produced gas in normal amount. Thus the temperature of 45°, whilst inhibiting growth, had not destroyed the organism.

(ii) The rate of denitrification became progressively slower [see also

Cranston and Lloyd, 1930]. This again was more marked at higher temperatures, the rate at 40° being 20 times less than that at 35°. This was probably due either to high mortality among the bacterial cells at this temperature, or to lethargy induced by this temperature. It may well be that at temperatures from 40° upwards, the bacterial cells develop into arthrospores and so are able to survive considerably higher temperatures.

C. *Other phases of bacterial growth.*

It has already been shown that the study by denitrification of the middle phases of bacterial growth can only be done with accuracy by using large quantities of broth culture and by making observations of volume changes at intervals not greater than half an hour.

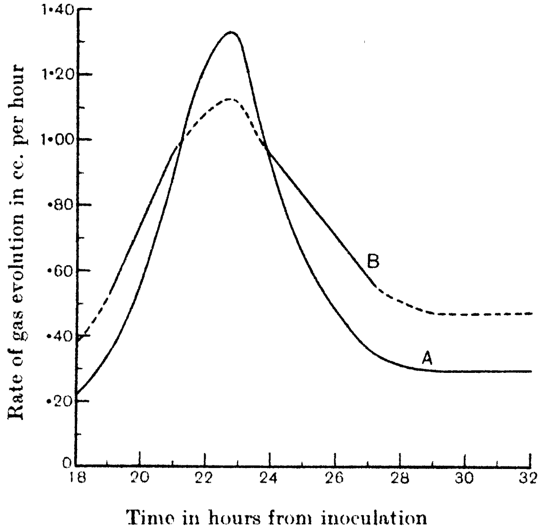

Fig. 10. A rate of denitrification curve *A* showing various phases of gas production which may be correlated with bacterial growth phases. The curve *B* is obtained by plotting the logarithms of these rates against the time. Two portions of this curve are straight, and represent the logarithmic growth phase and the logarithmic death phase respectively.

An experiment was carried out at 20·7° with 90 cc. of a broth culture containing 0·25 % KNO_3. This was contained in a boiling-tube fitted with a rubber stopper through which passed a glass tube connected to the capillary tube. After the broth was inoculated, the air above it was replaced by nitrogen. Gas began to evolve 17 hours later, and the volume was then measured at intervals of about 15 minutes for 12 hours. The average rate of denitrification was then calculated over successive periods of about half an hour and plotted against the mean time from inoculation. The result is shown in curve *A*, Fig. 10. The logarithm of the rate of denitrification was also plotted against the mean time, and the result is shown by the dotted curve *B*, Fig. 10.

The following phases are shown.

Logarithmic growth phase	from time	19 –21 hrs.
Phase of negative growth acceleration ...	„	21 –22·4
Maximum stationary phase	„	22·4–23·0
Phase of accelerated death	„	23 –25·5
Logarithmic death phase	„	23·5–27
Phase of slow decline	„	27 –29

From time 29 to 54, the rate of evolution of gas was almost constant at 0·3 cc. per hour, and then it deceased gradually until the 115th hour, after which no further gas was evolved.

It will be seen that in general the successive phases are of short duration; this is probably characteristic of growth in a medium containing nitrite because, in addition to the abundance of food supply available initially, oxygen is present in the nitrite in a well-distributed assimilable form. This results in rapid growth until the oxygen is used up, after which the decline is equally rapid. The intermediate phases are thus compressed, and the death phase becomes of the type usually associated with the presence of a germicide, *i.e.* it is logarithmic.

D. *The changes in acidity of the medium during growth.*

In the reduction of nitrates or nitrites to nitrogen two substances are formed which affect the acidity of the medium, viz. CO_2 and K_2O. These, of course, influence the p_H value in opposite directions. In the initial stages of the reduction, no alkali is formed at all, but oxygen is being consumed and presumably is combining with carbon in metabolism. At this stage there is a small increase in the acidity, the minuteness of which indicates that only a small proportion of the oxidised carbon is liberated into the broth as dissolved carbon dioxide; certainly no appreciable amount of CO_2 escapes in the gaseous state. At the later stage of the reduction, when nitrogen is evolved, K_2O is liberated, and forms KOH. The broth then rapidly becomes alkaline.

In pure aqueous solution the reduction of nitrate to nitrite results in considerably increased alkalinity, due to the hydrolysis of the nitrite. In broth, however, owing to its buffer action, the change in p_H for this reduction is found to be only 0·05, which is negligible compared with the other changes.

The following is a summary of many observations that have been taken on the changes in the acidity of a broth during the growth of a culture.

(i) *In absence of nitrites or nitrates.* When a sub-culture of Organism XIV is made into a broth of initial p_H 8·5 and free from nitrates or nitrites, the growth of the culture is accompanied by a slight increase in acidity to a p_H of about 8·2. After the usual phases of growth and decline have been completed, the senescent culture slowly, over a period of a few weeks, restores the medium to a p_H of about 8·6.

(ii) *In presence of* KNO_2. In a nitrite-broth culture of p_H 8·5, there is at first a slight increase in acidity, but when nitrogen is evolved, the p_H rapidly increases to a value of about 9·3. The final value depends on the initial concentration of the nitrite, for the greater the concentration up to 0·25 % the nearer to 9·3 is the final value.

(iii) *In presence of* KNO_3. In a nitrate-broth culture, the p_H first falls to 8·0, and then attains a final value of 8·2 to 8·8 according to the initial concentration of the nitrate. The lower final p_H value attained with nitrates as compared with nitrites is explained by the greater amount of oxygen available in the former (and consequent greater production of carbon dioxide) for the same amount of nitrogen produced.

SUMMARY.

1. The phases in gas production by actively denitrifying cultures have been studied by continuous measurements of the rate of gas production.

2. The conditions under which such rates of gas production can be directly correlated with rates of growth are discussed.

3. Some of the factors determining the duration of the lag phase of growth in organisms are considered.

4. By varying the amount of nitrate or nitrite experimentally, it is demonstrated that these substances are completely converted into some intermediate compound, which it is suggested is hyponitrite, before nitrogen is produced.

5. It is shown that one of the important factors in determining whether gas is produced or not, *i.e.* whether true denitrification occurs or not, is the initial concentration of the nitrate or nitrite.

6. The effect of temperature on growth is investigated.

7. In the interpretation of denitrification curves in terms of bacterial growth, it should be remembered that when all the factors are favourable to growth, the presence of nitrate or nitrite is specially conducive to rapid growth because oxygen is thus furnished throughout the medium in a most accessible manner. The multiplication of bacteria is therefore specially rapid as long as these substances last, after which the decline in the culture may be abnormally rapid, lack of oxygen being the principal limiting factor.

The above work was carried out in part at the Millport laboratory of the Scottish Marine Biological Association. Thanks are due to the Superintendent, Mr R. Elmhirst, for the facilities placed at the disposal of the authors.

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LXII. THE PRODUCTION OF HYDROGEN SULPHIDE BY ANIMAL TISSUES.

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DE REY PAILHADE [1888] demonstrated that yeast and various animal tissues could reduce sulphur to hydrogen sulphide, and ascribed this activity to the presence of a specific constituent which he called philothion. His views as to its precise nature have several times undergone change, but at one time at least [1894] he ranked it as an oxidation ferment. The view that an enzyme is concerned in the phenomena has been contested, among others, by Abelous and Ribaut [1903], and especially by Heffter, who [Heffter and Hausmann, 1904, 1908] decided that the reducing action of organs on sulphur depends upon the influence of SH groups. Heffter, as is well known, developed views which attached great importance to the thiol group of cysteine in the respiration of tissues.

Strassner [1910] showed that the presence of SH plays a part in the reduction of methylene blue by the tissues; but the extensive experiments of Thunberg and his colleagues, as well as those of other workers, have demonstrated how large a part is played by the dehydrogenases in the reduction of substances such as methylene blue. On the other hand, Hopkins [1921, 1922, 1925] finds that dehydrogenases in the presence of their substrates do not apparently reduce cystine or oxidised glutathione. The power of the tissues to reduce these depends upon other factors. It would seem of interest to decide whether nevertheless these enzyme systems can reduce free S itself. While it is certain that the presence of SH groups is largely responsible for the production of H_2S from S it is not yet certain that enzyme activity, calling upon other sources of hydrogen, takes no share in the process.

To decide this point was the aim of the present investigation. Experiments have been carried out to determine (1) whether the same proportionate relation exists between the amount of SH in the tissues and the amount of the H_2S produced therefrom as obtains when solutions of pure glutathione are similarly treated; (2) the influence of temperature and p_H on the process; (3) the influence of H-donator concentration; and (4) the effect of adding a dehydrogenase.

METHODS USED.

It is well known that a solution of H_2S, made acid with HCl, gives, on the addition of dimethyl-*p*-phenylenediamine sulphate and a small amount of ferric chloride, methylene blue. Upon this reaction a colorimetric method for the estimation of H_2S may be based. In applying this method in my own experiments I have employed not a colorimeter, but an extinctiometer as described by Moll. The procedure will be clear from the diagram (Fig. 1). A source of light (a 4-volt lamp) is placed between two thermopiles. Between the lamp and pile I is placed a trough containing the solution of which the heat adsorptive capacity is to be tested; between pile II and the lamp is placed an exactly similar trough containing water. The amount of heat which reaches the respective thermopiles and the corresponding potential produced varies of course with the adsorption exerted by the interposed solutions. The adsorption of the methylene blue solution under study is thus balanced against pure water as a control. The thermopiles are connected with a galvanometer as shown. *AB* and *CD* are symmetrically disposed resistances and the latter being adjustable is employed as a compensator.

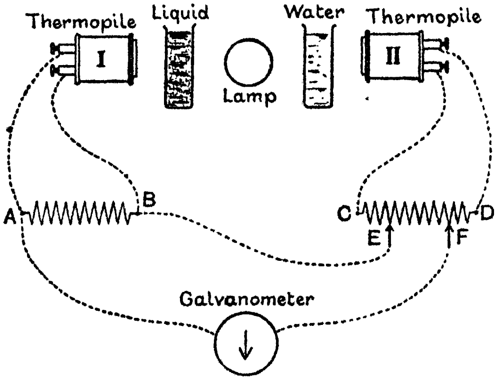

Fig. 1. Extinctiometer.

The apparatus is first adjusted by placing the lamp in such a position that both piles receive the same amount of radiation when both troughs are filled with water and the resistances equal. The galvanometer then registers no current. When in trough I the water is replaced by the coloured solution there is greater heat adsorption and the consequent diminution of the current from pile I is registered by the galvanometer. Equilibrium is then restored by shifting the contacts of *CD*. Decrease of the distance between *C* and *D* is proportional to a decrease in the thermoelectric current from pile II. The reading in ohms on the scale of *CD* gives, when the apparatus is calibrated, a direct measure of the concentration of methylene blue in trough I.

In order to test the method, varying known concentrations of methylene blue were placed in trough I and the readings on the compensator scale were plotted against these. Duplicate determinations agreed well (Table I) and the results yielded a smooth curve (Fig. 2).

Table I.

cc. of methylene blue sol. in 20 cc. water	Ohms
0·25	4·6; 4·6
0·50	7·2; 7·1
1·00	10·0;
2·00	12·8; 12·8

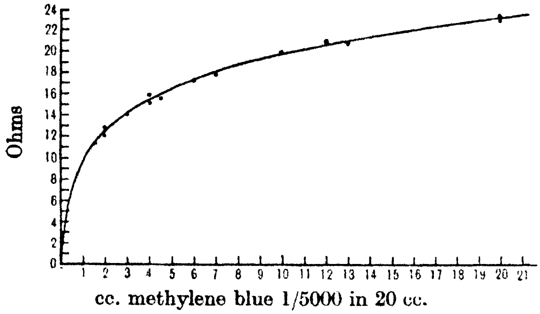

Fig. 2. Extinction curve of methylene blue.

There is, however, no relation between the extinctiometric values of solutions of pure methylene blue and the solutions containing HCl as prepared from H_2S on the lines described. The spectrum of methylene blue has two bands in the red while that of its hydrochloride has but one; that on the boundary between red and yellow being absent from the latter. The colour of a solution containing HCl has therefore an element of orange. Since yellow absorbs more heat than violet less heat is transmitted by a given concentration of methylene blue when HCl is present.

The results given in Table II show this effect, the figures being given in the form of the compensator readings. It will be seen that the reagents added for the production of methylene blue from the H_2S have only a negligible heat absorption.

Table II.

Methylene blue 1/5000 cc.	Water cc.	HCl. cc.	Reagents cc.	Ohms
5	10·5	0	0	19·6
5	8	2	0·5	29·9
5	8·5	2	0	29·0
5	10	0	0·5	20·3
0	13	2	0·5	0·7

In order to calibrate the apparatus for use it was therefore necessary to construct a curve from methylene blue solutions as obtained by adding the reagents to H_2S solutions of known concentration, the procedure being the same as in the actual experiments. The H_2S solutions were made by diluting a solution very accurately titrated with iodine. The curve thus obtained is given in Fig. 3.

As a further test of the consistency of the results determinations by the method were made on each of two solutions which were then mixed, and determinations again made on the mixture. It will be seen (Table III) that the results are consistent.

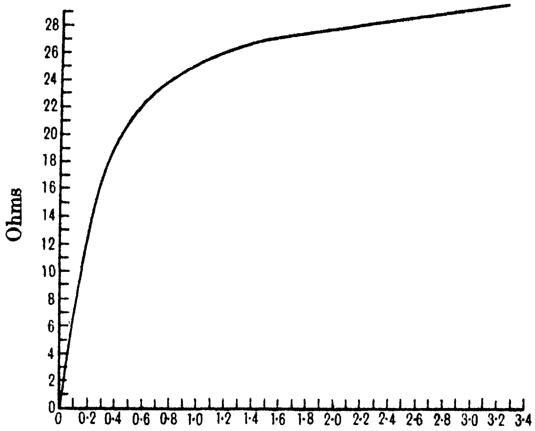

Fig. 3. Calibration curve for H_2S.

Table III.

Solution	H_2S estimated mg.	Average mg.	H_2S determined in mixture mg.
a	0·62	0·405	0·42
a′	0·19		
b	0·91	0·72	0·74
b′	0·53		
c	0·81	1·04	1·05
c′	1·28		
d	0·28	0·20	0·20
d′	0·12		
e	0·09	0·07	0·065
e′	0·05		

A number of experiments were made to determine the effect of temperature changes upon the result. It was found that within the ordinary range of room temperatures the effect was small though not negligible. It is best to work near to the temperature at which the apparatus is calibrated. Keeping the methylene blue solutions for 24 hours before making the estimation was found to have no effect upon the results.

THE YIELD OF H_2S WHEN SULPHUR IS BROUGHT IN CONTACT WITH GLUTATHIONE.

Hopkins has mentioned incidentally that reduced glutathione when shaken with sulphur yields H_2S. I have to thank Sir F. G. Hopkins for a supply of that substance sufficient for the following experiments.

I first set out to determine whether the thiolpeptide yields amounts of H_2S proportionate to the concentration of SH in solution. Solutions of various concentrations were made, the SH being determined by titration with $N/100$ iodine. The p_H of all solutions was adjusted to 7·6. To each was added a measured quantity of a 2 % solution of colloidal sulphur. The solutions were placed in bottles which were evacuated and placed in a thermostat for 2 hours. At the end of a given period the H_2S was in each case drawn over by a stream of air which had been washed through lead acetate solution into a known volume of $N/10$ NaOH. To the solutions thus obtained $N/10$ HCl was added in amount equivalent to the NaOH employed. Concentrated HCl (2 cc.) was then added, 0·5 cc. of the solution of dimethyl-*p*-phenylenediamine sulphate, and finally two drops of ferric chloride solution. Estimations of the methylene blue so obtained were made in the extinctiometer apparatus (Table IV). The first two solutions were colourless.

Table IV.

SH in solution (mg.)	H_2S produced (mg.)	SH in solution (mg.)	H_2S produced (mg.)
1·52	0	3·04	0·24
1·88	0	4·86	0·60
2·43	0·14	5·25	0·63

The production of H_2S appears to require a certain minimum of SH before it becomes evident. The remaining figures are few, but if plotted they show a linear relation and suggest that 1 mg. SH after 2 hours' contact of the glutathione with sulphur yields 0·2 mg. H_2S.

THE RELATION BETWEEN SH-CONTENT OF TISSUES AND H_2S PRODUCTION WITH SULPHUR.

The literature contains a large number of estimations of the SH content of tissues. It would take, however, too much space to summarise them here. The following figures were obtained by me in the course of this research, using Tunnicliffe's method [1925]. They are average values and are given in terms not of glutathione, but of SH (mg. per 100 g. tissue).

Liver: (cow) 36·4; (dog) 52·5; (rabbit) 45·1.
Kidney: (dog) 24·5; (rabbit) 21·6.
Thymus: (calf) 35·2.
Lung: (cow) 17·2; (dog) 9·5.

For the estimation of the yield of H_2S by tissues in contact with sulphur the same technique was employed as that described above. Under the circumstances of these experiments the tissues without added sulphur yielded no H_2S (Tables V and VI).

Liver was chiefly employed. In the case of smaller animals this was the only organ yielding enough material for a series of observations, and it easily yields uniform preparations. The degree of subdivision of a tissue is not without effect upon the rate of H_2S formation. All values given represent the mean of two determinations; if these differed by more than 10 %, the result was rejected; the difference was usually much smaller.

The urethane narcosis consisted in the injection intraperitoneally of a 25 % solution of urethane in water, 4 g. per kg.

The relation between amount of tissue (or amount of SH contained in it) and H_2S evolved is in all cases strictly linear.

Table V.

Exp. no.	Weight of tissue (g.)		SH content (mg.)	H_2S evolved (mg.)
1	Liver	0·5	—	0
		1·0	—	0·38
		1·5	—	0·81
		2·0	—	1·23
2	Liver	0·5	—	0·74
		1·0	—	1·38
		1·5	—	2·00
3	Liver	0·5	1·16	0
		1·0	2·32	0·46
		1·5	3·48	1·06
		2·0	4·64	1·73
4	Kidney	0·5	1·03	0
		1·0	2·06	0·47
		1·5	3·09	0·80
		2·0	4·12	1·04
5	Liver	0·5	0·88	0
		1·0	1·76	0·34
		1·5	2·64	0·64
		2·0	3·52	0·90

Further experiments were carried out upon various organs from different animals in order to determine the yield of H_2S from the existing concentration of SH in the tissue in question (Table VI).

If in these experiments only the amount of SH and the quantity of H_2S produced be considered, it will be evident that no parallelism or regularity is shown. This is in agreement with the statements of Hausmann.

In order to compare organs concerning their production of H_2S, experiments were carried out with the organs of the same animal, yielding the results given in Table VII.

For the comparison of the H_2S production per 1 mg. SH of the different organs, the experiments in which the animal was not narcotised are the most reliable. These also show that the values are not in agreement (Table VII).

From the figures the conclusion may be drawn that organs from larger animals such as the cow, dog and pig produce less H_2S than organs from small animals.

Hopkins [1921, 1922, 1925] finds in the tissues of small animals in all circumstances a higher reducing power than in the tissues of larger animals.

These experiments show that a marked difference exists between the H_2S formation by tissues and by reduced glutathione. The action of microbes can be excluded in the first case, for toluene was added and the period was too short. The rate of H_2S production was measured by starting a series of equal

Table VI.

Exp. no.	mg. SH	mg. H_2S	Remarks
			LUNG.
2*a*	1·20	0·40	Cat, organ fresh, no narcosis
3	1·23	0·48	Rabbit, organ 1 day old, 1 hour ether narcosis
4	1·60	0·87	Rabbit, organ 1 day old, 1 hour ether narcosis
5	1·64	0·60	Rabbit, organ 1 day old, urethane narcosis
6	1·79	0·95	Rabbit, organ 1 day old, urethane narcosis
7	2·49	0·12	Dog, organ 1 day old, 2 hours' ether-chloroform narcosis
8	2·75	1·11	Rabbit, organ 1 day old, 1 hour ether narcosis
9	3·32	0·15	Dog, same as in exp. 7
10	3·50	0·68	Rabbit, organ fresh, no narcosis
11	7·6	0	Cow, organ 1 day old
12	9·5	0	Cow, same as in exp. 11
13	12·62	0·28	Cow, organ 2 days old
			LIVER.
1	1·59	0	Rabbit, organ 1 day old, 1 hour ether narcosis
1*a*	1·64	1·35	Cat, organ fresh, no narcosis
2	3·17	0·69	Rabbit, organ 1 day old, urethane narcosis
3	3·19	0·64	Rabbit, organ 1 day old, 1 hour ether narcosis
4	3·21	0·28	Rabbit, organ 1 day old, ether narcosis
5	4·23	0·82	Rabbit, organ fresh, no narcosis
6	5·9	1·15	Rabbit, organ 1 day old, narcosis?
7	6·38	0·61	Rabbit, organ 1 day old, 1 hour ether narcosis
8	7·44	0·27	Dog, organ 1 day old, ether-chloroform narcosis
9	7·9	1·39	Rabbit, organ 1 day old, narcosis?
10	9·9	1·46	Rabbit, organ 1 day old, narcosis?
			KIDNEY.
1	1·02	0	Rabbit, organ 1 day old, urethane narcosis
2	1·34	0·73	Rabbit, organ 1 day old, 1 hour ether narcosis
3	1·51	0·72	Rabbit, organ 1 day old, 1 hour ether narcosis
4	1·61	0	Cow, organ fresh
5	1·65	0·50	Rabbit, organ 1 day old, ether narcosis
5*a*	1·70	0·71	Cat, organ fresh, no narcosis
6	2·18	0·02	Dog, organ 1 day old, ether-chloroform narcosis
7	2·19	1·07	Rabbit, organ fresh, no narcosis
8	2·20	0·54	Dog, organ 1 day old, 4 hours' ether-chloroform narcosis
9	2·48	0·26	Dog, organ 1 day old, ½ hour chloroform narcosis
10	2·72	0·22	Dog, organ 1 day old, 4 hours' ether-chloroform narcosis
11	3·16	0	Pig, organ 3 days old
12	3·30	0·05	Pig, organ fresh
13	3·70	0·83	Dog, organ 1 day old
14	4·26	0·29	Cow, organ 2 days old
15	5	0·43	Pig, organ 2 days old
16	6·57	2·53	Pig, organ 1 day old
			THYMUS.
1	1·19	0·25	Calf, organ 1 day old
2	2·39	0·45	Calf, organ fresh
3	3·30	0·39	Calf, organ 5 days old
4	12·82	1·37	Calf, organ 1 day old
5	13·06	1·10	Calf, organ 1 day old
			SPLEEN.
1	2·12	0·13	Cow, fresh
2	4·52	0	Cow, organ 1 day old
3	8·07	0	Cow, organ 2 days old
4	11·30	0	Cow, organ 1 day old
5	1·50	0	Rabbit, fresh, no narcosis
6	1·99	0·18	Cat, fresh, no narcosis

Table VII.

Exp. no.	Organ	mg. SH	mg. H_2S	mg. H_2S to 1 mg. SH	Remarks
1	Kidney	1·34	0·73	0·54	Rabbit, 1 hour ether narcosis
	Lung	1·23	0·48	0·39	
2	Liver	3·17	0·60	0·19	Rabbit, urethane narcosis
	Kidney	1·57	0·13	0·082	
	Lung	1·79	0·95	0·52	
3	Liver	1·59	0	0	Rabbit, 1 hour ether narcosis
	Liver	6·38	0·61	0·095	
	Kidney	1·51	0·72	0·47	
	Lung	1·60	0·87	0·54	
	Lung	0·80	0	0	
4	Liver	3·21	0·28	0·087	
	Kidney	1·65	0·50	0·303	
	Lung	1·15	0·09	0·078	
5	Brain	5·14	0·90	0·175	Rabbit, no narcosis
	Liver	4·32	0·82	0·189	
	Lung	3·50	0·68	0·194	
	Kidney	2·19	1·07	0·488	
	Spleen (1·5 g.)				
6	Liver	1·64	1·35	0·823	Cat, no narcosis
	Kidney	1·70	0·71	0·419	
	Lung	1·20	0·40	0·333	

quantities of tissues and measuring the amount of H_2S produced after a certain time. The curves of Fig. 4 show some examples: in the first hour or the first half hour much H_2S was produced, and the longer the period the less was produced.

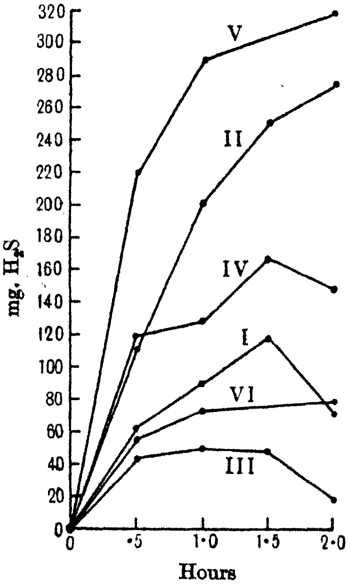

Fig. 4. Rate of production of H_2S by liver.

I Liver (cat) 1·56 mg. SH per 1 g.
II Liver (cat) 1·83 ,,
III Liver (dog) 3·58 ,,
IV Liver (cat) 1·16 ,,
V Liver (cat).
VI Liver (cat) diluted.

Various factors which might throw light on the nature of the action were now investigated.

Temperature.

The tissues were treated in the usual way by rasping or cutting. The quantities were weighed and the bottles were placed in the boiling water-bath. The temperature in the bottles averaged between 70 and 80°. The amount of SH was also determined in heated and non-heated tissue by Tunnicliffe's method. The coarseness of the tissue is important for the decrease in SH. The duration of heating also is of influence. The tissue used for H_2S production must always be of equal fineness (Table VIII).

Table VIII.

	mg. SH	mg. H_2S
1 g. liver, not heated	4·15	2·10
10 min. at 70–80°	3·05	1·10
2 g. liver mixture, not heated	2·9	0·69
5 min. at 70–80°	2	0·45
10 min. at 70–80°	0·70	0

In an SH solution obtained by treating tissue with trichloroacetic acid the amount of SH decreased in the same way in proportion to duration of heating and to temperature (Table IX).

Table IX.

	cc. *N*/100 Iodine
5 cc. SH solution, determined immediately	0·95
after 5 min. at 70–80°	0·83
after 10 min. at 100°	0·71

The H_2S production therefore is decreased by heating and dissolving, because the SH group is decomposed. This can be demonstrated in a simple way, by bringing together the solution, or the tissue rasped and diluted with a buffer solution, and sulphur in a bottle and introducing a lead acetate paper. The bottle is evacuated and placed in boiling water. The longer the bottle remains in water, the more H_2S is produced, and the SH content of the tissue or solution decreases. In one case 3 cc. SH solution contained 2·76 mg. SH when not heated; after boiling for 8 minutes, 2·16 mg.; in another case the non-heated SH solution contained 1·2 mg. SH; after boiling for 8 minutes it contained 0·75 mg. The same is true for tissues. When 1 and 2·1 cc. of tissue with lead paper are placed in boiling water for 1 and 2 minutes copious production of H_2S is noticed. When the same quantities without lead paper are heated for the same length of time and the lead acetate paper afterwards introduced, and the bottles then evacuated and placed again in boiling water for 1 and 2 minutes, H_2S production is still evident in the first case, but not in the second. The SH had therefore been decomposed by heating. Abelous and Ribaut also observed a temporary increase of H_2S production on heating.

Tissue yields H_2S on boiling only when S is added.

The rates of decrease of SH groups and of H_2S production are not proportional, as the H_2S production decreases much more rapidly. This agrees with the observation of Tsubura [1925] and Meyerhof [1926, 1928].

Influence of p_H.

In my experiments I used buffer solutions of primary and secondary phosphate (Clark). Equal quantities of tissue were brought together with 1 cc. of a phosphate buffer solution (Clark).

Some of the results obtained are shown in Fig. 5. The absolute heights of the curves are not comparable, but this is unnecessary since, as remarked already, the duration of the experiments varied. From these curves may be deduced that generally H_2S production does not take place in a system at p_H 4. With increasing alkalinity H_2S production starts or increases and reaches its maximum at p_H 7·8. If the alkalinity increases still more the production decreases again. No error is caused by absorption of H_2S by the buffer.

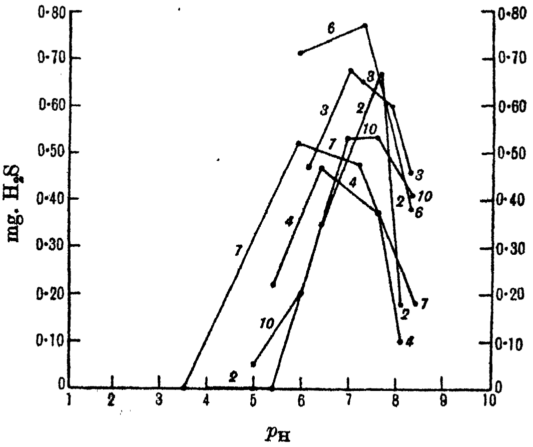

Fig. 5. Influence of p_H on H_2S production of tissues.

2 Liver 0·61 mg. SH.
3 Liver 0·61 „
4 Kidney 0·845 „
6 Kidney 1·66 „
7 Kidney.
10 Liver.

Influence of H-donators.

Concerning glutathione, Hopkins [1922] states that neither succinate nor citrate accelerates the reduction of glutathione. Reduced glutathione does not act as a H-donator in Thunberg's sense. This is deduced by Hopkins from equilibrium determinations in which no relation could be found between the quantity of methylene blue reduced and the quantity of H-donator changed.

In preliminary experiments I used a 1 % solution of potassium succinate and observed that this caused the H_2S production in various organs to increase (Table X).

Table X.

	mg. SH to 1 g. tissue	mg. H_2S produced
4 g. lung tissue	3·32	0·15
+ 1 cc. 1 % K_2 succinate	3·32	0·51
1 g. liver	3·17	0·69
+ 1 cc. 1 % K succinate	3·17	1·13
1 g. liver	—	1·65
+ 1 cc. 1 % K succinate	—	1·73
1 cc. kidney mixture	0·51	0
+ 1 cc. 1 % K succinate	0·51	0·27
2 g. kidney	—	0·29
+ 1 cc. 1 % K succinate	—	0·44
1 g. lung	1·64	0·58
+ 1 cc. 1 % K succinate	1·64	0·02
2 g. kidney	—	0·29
+ 1 cc. 1 % K succinate	—	0·47
5 g. spleen	11·30	0
+ 1 cc. 1 % K succinate	11·30	0·23

Those preliminary experiments therefore show, contrary to the results of Hopkins obtained with glutathione, that H_2S production is influenced by hydrogen donators. If the tissue alone produces a relatively large amount of H_2S the effect of adding a hydrogen donator is smaller than in a case in which the H_2S production as such is slight. The experiments cannot be compared quantitatively since the times were not the same.

It is possible that the greater or less effect of the added hydrogen donator is dependent on the amount of natural hydrogen donator contained in the tissue at the moment of the experiment.

In addition to succinate, tartrate, citrate and malate were also investigated. Thunberg [1918, 1920] obtained with these salts an acceleration of the decoloration of methylene blue and drew the conclusion that they might act as hydrogen donators. The solutions used by me were 0·1 *N* accurately neutralised to phenol red at p_H 7·6. To the tissue 1 cc. of the donator was added.

The quantity of H_2S produced was compared with the quantity produced by tissue + 1 cc. phosphate buffer at p_H 7·6 or with 1 cc. water at p_H 7·6 (mixture of tap water at p_H 7·8 and distilled water at p_H 6·2) (Table XI).

Table XI.

	mg. H_2S					
1 cc. *N*/10 tartrate + tissue	0·215	—	—	0·41	—	0·97
1 cc. *N*/10 citrate + tissue	0·79	0·70	0·77	1·07	0·90	2·42
1 cc. *N*/10 malate + tissue	0·19	—	0·32	0·45	0·32	0·91
1 cc. *N*/10 succinate + tissue	0·21	0·36	0·36	—	—	0·835
1 g. liver + 1 cc. buffer p_H 7·6	0	0	0	0·37	0·13	—
1 g. kidney + 1 cc. buffer p_H 7·6	—	—	—	—	—	0·68

Tartrate, malate and succinate have apparently an equal ability to act as hydrogen donator, but citrate yields without exception a much larger H_2S production. Controls without tissue gave negative results.

If only the hydrogen directly combined with C acts as a donator, as Thunberg assumes, the larger number of H atoms (4 in citrate and succinate, 2 and 3 in the tartrate and malate) cannot be the cause. But it is possible that the H of the OH group in citric acid is more mobile than the H in the OH group in malic and tartaric acids. It is remarkable that Tsubura was not able to demonstrate this action of citrate, but observed a renewed power of reduction in washed muscle pulp after adding potassium succinate.

The few experiments carried out by me with reduced glutathione and hydrogen donators also showed an increased H_2S production (Table XII).

Table XII.

		mg. SH
Glutathione + buffer solution (p_H 7·6)	+ S	0·04
+ citrate $N/10$	+ S	0·18
+ buffer solution (p_H 7·6)	+ S	0·35
+ succinate $N/10$	+ S	0·42

In order to investigate the influence of the concentration of the hydrogen donator various dilutions of citrate and malate were used.

M/10 citric acid (21·01 mg. to 1 cc.)
M/10 malic acid (13·4 „ „)

From both solutions 5 dilutions were prepared, from the citrate 10·5, 5·25, 2·625, 1·31 and 0·65 mg., from the malate 6·7, 3·3, 1·65, 0·82 and 0·41 mg. in 1 cc.

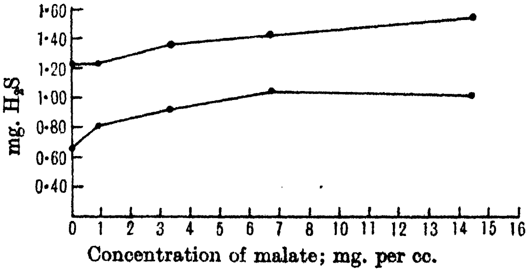

Fig. 6. Effect of malate as H-donator.

The curves (Figs. 6 and 7) show the H_2S production in these concentrations with different tissues. In the first place the steeper course of the curves after adding citrate is remarkable, *i.e.* the increased H_2S production in comparison with that after adding malate.

In the second place it is evident that, if the tissue as such has produced much H_2S, adding a small amount of donator has little or no influence.

Thirdly, both donators, but citrate more clearly than malate, show a decrease in H_2S production when the donator concentration is increased. The curve is parabolic. The optimum activity is situated at 6/20 *M* to 1/10 *M* for both salts.

A single experiment was made with citrate at higher concentration, which showed that the largest amount of H_2S was liberated at such a concentration (Table XIII).

An inhibition of H_2S production by adding donator was never observed if the p_H were carefully adjusted.

Table XIII.

		mg. citrate	mg. SH	mg. H_2S
1 g. liver + 1 cc.	buffer p_H 7·6	0	1·51	0·60
liver +	citrate $M/2$	100·5	—	1·23
liver +	citrate diluted × 2	50·25	—	1·58
liver +	citrate diluted × 4	25·15	—	1·72
liver +	citrate diluted × 8	12·6	—	1·80

Influence of dehydrogenase.

In order to investigate the influence of dehydrogenase on the H_2S production of tissues a solution was prepared by Ohlson's method [1921]. 0·5 to 1 kg. of lean horseflesh, freed from fat and fascia, was ground three times and washed with 0·25 % NaCl solution until colourless, filtered through a cloth and pressed. The residue was shaken for an hour with an equal volume of 2·4 % Na_2HPO_4 solution and centrifuged.

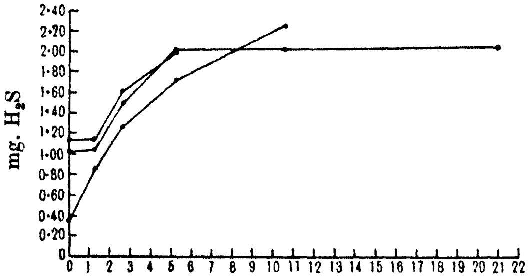

Fig. 7. Effect of citrate as H-donator.

The enzyme solution had a much lower activity in presence of sulphur than tissue and also gave a much fainter nitroprusside reaction. In these experiments I used Thunberg bottles in which a lead acetate paper was placed, the bottles being left in the thermostat for 20 hours. These experiments were not quantitative but the difference in hue of the lead papers afforded an approximate indication.

1. *Temperature.* Heating at 65° for 10 minutes has little influence (in spite of the fact that according to Thunberg all dehydrogenases are inactivated at 60°). If the tubes be placed in boiling water after evacuation and introduction of the lead acetate paper, the papers are immediately coloured black; as in the case of tissue boiling causes much H_2S to be produced quickly, the quantity of SH is exhausted and therefore after boiling but little H_2S can be produced.

2. *Effect of* p_H. A marked H_2S production was observed at p_H 5, sometimes larger, sometimes smaller, than at p_H 7·6. At p_H 8·3 the H_2S production is positive but less marked.

3. *H-donators.* As is the case of the tissue experiments addition of citrate promotes H_2S production. Succinate also promotes H_2S production. The effect of adding hydrogen donators is only evident if the enzyme solution be not too concentrated.

Those experiments show that the enzyme (dehydrogenase) solution used had the same activities as tissue or glutathione but to a much less pronounced extent.

If the enzyme solution be added to normal tissue, little increase in H_2S production can be noticed in 1 to 1½ hours.

SUMMARY.

1. Three different sources of SH have been used.

(1) Tissues.
(2) SH solutions (glutathione or organ extracts).
(3) A dehydrogenase solution.

All these sources yield H_2S after addition of S. In reduced glutathione, as well as in tissue, H_2S production is during a certain time directly proportional to the quantity of SH present. Since all tissues contain SH, the H_2S production can be explained undoubtedly by the purely chemical process:

$$2G.SH + S = G.S.S.G. + H_2S.$$

The H_2S production by tissues is much more marked than by the pure SH compound, when both contain the same amount of SH. This is not necessarily due to enzyme activity, but can be explained by the possibility that the SH group in tissues is more active than in the pure compound. Catalytic factors (iron catalysis, as mentioned by Warburg and Sakuma) may play a more important part in the tissues than in the pure SH preparation.

A solution of dehydrogenase (an enzyme which may be expected to yield H_2S when brought together with S) produces very little H_2S. If the enzyme be added to normal tissue no increase in H_2S production results. It is a remarkable fact that the organs of small animals have a higher ability to produce H_2S than those of large animals, the amount of SH being the same.

2. With all three sources of SH the influence of various factors was investigated.

(1) *Temperature.* Increase of temperature, even up to boiling point, results in promotion of H_2S formation and decrease of the SH content of tissue. This phenomenon cannot be explained by enzyme activity; on the contrary, an enzyme should be destroyed at 100°. If the H_2S production of heated tissue is determined, it appears that the H_2S production decreases more quickly than the SH content. The heating therefore probably affects the other factors that are concerned with H_2S production.

(2) p_H. In a strongly acid or alkaline system the H_2S production is small or nil. The optimum is at about the neutral point. This holds for glutathione as well as for tissues and enzyme solutions.

(3) *H-donators.* Addition of H-donators results in an increase of H_2S production. If the tissue as such is able to produce a large amount of H_2S with S, the addition of H-donators is of little effect. Citrate gives rise in all three cases to the largest increase in H_2S production. The donators have an optimum concentration between *M*/10 and *M*/20.

3. Since H_2S production may take place under circumstances in which enzymes are inactive and since an enzyme solution does not produce more H_2S than corresponds with its content of SH, I believe the conclusion to be justified that H_2S production in living tissue after addition of sulphur is not due to enzyme activity.

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LXIII. A REDETERMINATION OF THE TITRATION DISSOCIATION CONSTANTS OF ARGININE AND HISTIDINE WITH A DEMONSTRATION OF THE ZWITTERION CONSTITUTION OF THESE MOLECULES.

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We were prompted to undertake the present redetermination by the consideration that certain values recently put forward are in serious conflict with those previously accepted. (1) In the first place, Simms [1928] has given constants for arginine, and, although he presents no experimental evidence in their support, and notwithstanding the fact that they diverge widely from earlier values, he nevertheless uses them as the basis for postulating that arginine exists in protein in the form of some unknown precursor, termed "prearginine." We do not propose to discuss here the validity of Simms's assumption that a knowledge of the p_k value of a given group in an amino-acid enables one as yet to fix its value when it is combined in a complex protein molecule. Our purpose is merely to record results which confirm the earlier constants as opposed to those used by Simms. (2) In the case of histidine, Hirsch's [1924] values and curves differ from Harris's [1923, 1] conclusions in showing only one acid and one basic group instead of one acid and two basic groups. Further, the magnitude of the two p_k constants given by Hirsch are not in particularly good agreement with the earlier values. In our redetermination we have confirmed the earlier conclusions, both as to the orders of magnitude, and also the number, of the constants. The issue is of some interest because several workers have made use of Harris's description of tyrosine and histidine as diacid-monobase and dibase-monoacid ampholytes, respectively, to account for the acid- and base-binding power of proteins [*e.g.* Greenberg and Schmidt, 1924; Cohn and Berggren, 1925].

While carrying out the redeterminations we also proceeded to obtain experimental evidence of the zwitterion constitution of arginine and histidine.

I. Arginine.

From conductivity measurements of arginine salts, Kanitz [1906] first attempted to calculate the ionisation constants; but, "as pointed out by Harris [1923, 1], Kanitz's tabulation of his results contains misprints which completely distort some of his actual conclusions" [Hunter and Borsook, 1924], two out of the three constants being incorrectly set out. Harris [1923, 1], from a revision of the conductivity data of Kanitz, as well as his own colorimetric, electrometric and p_H-end-point determinations, described the predicted titration curve, consisting of two well separated components having mid-points at p_H 2·3 and 9, plus a third value in an inaccessibly alkaline zone approaching p_H 13–14; later a direct titration curve led him to the very accurately calculated value of 2·17 for the first component [Harris, 1925][1]. Hunter and Borsook [1924] and also Hirsch [1924] obtained titration curves giving p_k values very similar to the above, viz. 2·17, 9·10 and 12·84; and 2·07, 9·18, —, respectively. Simms [1928], on the other hand, has cited values of 2·29, 9·64, and 8·15, the last being described as relating to the =NH group.

EXPERIMENTAL.

1. *Titration in water.*

Several specimens of free arginine base, from two different sources, were used and the technique employed was that described in earlier papers [Harris, 1923, 1, 2; 1925], which has since been generally adopted for determining dissociation constants in place of conductivity and other methods. The arginine was dissolved in water at approximately 0·1 *N* dilution and placed in the small specimen tube which was employed as titration vessel, and in which an atmosphere of hydrogen sufficed to prevent any absorption of CO_2 in the alkaline zone of the determination. The vessel was placed in a large box electrically regulated to a constant temperature, p_H measurements being made by use of the following: hydrogen electrode of the Hildebrand-Cole pattern, saturated calomel half-cell, agar-KCl bridge, and Cambridge and Paul portable potentiometer. As absolute standard 0·1 *N* hydrochloric acid was adopted, for which a p_H value of 1·07 was assumed; and the calomel cell was standardised against it. In order to diminish the magnitude of the blank correction in the titrations, and hence increase the accuracy of the determination [Harris, 1923, 1, p. 460], *N* HCl and NaOH were used rather than 0·1 *N*, with a microburette graduated in 0·01 cc.

From the p_H resulting after each addition of NaOH or HCl, p_k values were calculated by the Henderson-Hasselbalch approximation equations, as modified to allow for the blank correction for free NaOH or HCl [Harris, 1923, 1].

$$p_k = p_H - \log \frac{[\mathrm{Na}] - [\mathrm{OH}^-]/\gamma}{[\text{total amino-acid}] - ([\mathrm{Na}] - [\mathrm{OH}^-]/\gamma)}$$

$$p_k = p_H + \log \frac{[\mathrm{Cl}] - [\mathrm{H}^+]/\gamma}{[\text{total amino-acid}] - ([\mathrm{Cl}] - [\mathrm{H}^+]/\gamma)}.$$

Each value of γ, required in solving the above formula, was calculated on the basis of the total concentration of sodium (or chloride) present at the reading in question, and was arbitrarily assumed to be the same as for an equal concentration of NaOH (or KCl) in water alone—approximations giving results sufficiently accurate for the present purpose [Harris, 1925]. These values of γ for NaOH and KCl were taken from the result of Harned [1925] and Noyes and McInnes [1920], respectively. If great precision is required in the very acid part of the curve, $p_H < 2{\cdot}2$, it would be more accurate to take somewhat higher values for γ, *i.e.* approximating more towards γ for HCl.

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[1] The approximate p_K 9 value was fixed by determining its p_H-end-point, which was found to be p_H 7 [Harris, 1923, 1, pp. 453, 482, 451] and also electrometrically [Harris, 1923, 2, p. 3300]; whilst the highly alkaline value was based on the observation that no measurable alkali was needed when titrating arginine to the standard end-point p_H 11·5, satisfactory for other amino-acids [Harris, 1923, 1, pp. 479].

The experimental results and values derived therefrom are tabulated below.

Table I. *Titration of arginine with NaOH at 23°**.

0·1050 g. dissolved in 5·0 cc. of CO_2-free water and titrated with 1·08 *N* NaOH.

			(*d*)				
(*a*) E.M.F.	(*b*) p_H	(*c*) [H$^\bullet$]	cc. of 1·08 *N* NaOH added	Expressed as *N*/1 NaOH	(*e*) Total vol.	(*f*) [NaOH] $=d/e$	(*g*) γ corresponding with *f*
868·0	10·53	$0{\cdot}30 \times 10^{-10}$	0·000	0·000	5·00	—	—
898·5	11·05	$0{\cdot}89 \times 10^{-11}$	0·022	0·024	5·02	0·0046	0·92
926·0	11·52	$0{\cdot}30 \times 10^{-11}$	0·050	0·054	5·05	0·0107	0·92
946·0	11·85	$0{\cdot}14 \times 10^{-11}$	0·086	0·093	5·09	0·0183	0·88
955·0	12·00	$1{\cdot}00 \times 10^{-12}$	0·115	0·125	5·12	0·0244	0·88
963·0	12·14	$0{\cdot}73 \times 10^{-12}$	0·150	0·162	5·15	0·0315	0·86
979·0	12·40	$0{\cdot}40 \times 10^{-12}$	0·260	0·283	5·26	0·0538	0·82
987·5	12·56	$0{\cdot}28 \times 10^{-12}$	0·360	0·390	5·36	0·0728	0·78
994·0	12·66	$0{\cdot}22 \times 10^{-12}$	0·470	0·509	5·47	0·0931	0·77
1009·0	12·91	$0{\cdot}12 \times 10^{-12}$	0·850	0·920	5·85	0·157	0·75

(*h*) [OH′] $=K_W/[H^\bullet]$	(*i*) [Free NaOH] $=[OH']/\gamma$	(*j*) [Combined NaOH] $=f-i$	(*k*) cc. of combined NaOH, 1·0 *N* $=j \times e$	(*l*) Equivs. of combined NaOH	(*m*) $\log a/(1-a)$	(*n*) p_k
—	—	—	—	—	—	—
0·00124	0·00135	0·0033	0·0164	—	—	—
0·00367	0·00398	0·0067	0·0339	0·029	+1·51	13·03
0·00786	0·00893	0·0094	0·0479	0·053	+1·25	13·10
0·0110	0·0125	0·0119	0·0609	0·076	+1·09	13·09
0·0151	0·0176	0·0139	0·0712	0·092	+0·99	13·13
0·0275	0·0355	0·0183	0·096	0·125	+0·84	13·24
0·0393	0·0504	0·0224	0·120	0·176	+0·67	13·23
0·0500	0·0650	0·0281	0·154	0·230	+0·52	13·18
0·0916	0·122	0·035	0·209	0·322	+0·32	13·23

* In view of the fact that specimens of arginine base may contain variable amounts of water of hydration and possibly also traces of combined acid the following method was adopted to convert actual amounts of HCl or NaOH added into true equivalents of HCl or NaOH, *i.e.* equivalents corrected for normality of the arginine preparation. The p_H values (ordinates) were plotted against the HCl (or NaOH) actually added (abscissae), and the scale for the abscissae was then inserted as equivalents, so as to show exactly one unit between the two well marked points of inflection which occur at about p_H 6 and 11, representing monohydrochloride and free base respectively. The values on this scale of equivalents are used in the above table.

The specimen of arginine on which the above results were obtained gave on combustion: N = 30·2 % (by titration, 4 × amino-N = 31·6 %; theory for $C_6H_{14}O_2N_4$ = 32·18).

A duplicate titration curve was determined upon a second specimen of arginine, derived from the same source (Hoffmann la Roche), and gave closely concordant values (not shown).

A specimen of "arginine" obtained from Pfanstiehl on the other hand, although described as of 100 % purity, required only 81·3 % of the theoretical amount of HCl to titrate one group; gave irregular p_k curves; and on analysis showed C = 38·47 %, H = 7·90 %, N = 26·57 % (theoretical for $C_6H_{14}O_2N_4$: C = 41·34 %, H = 8·10 %, N = 32·18 %). Its titration values are therefore neglected.

Table II. *Titration of arginine with HCl at 23°.*

0·1050 g. dissolved in 5·0 cc. of CO_2-free water and titrated with 1·0 *N* HCl.

(*a*) E.M.F.	(*b*) p_H	(*c*) [H˙]	(*d*) cc. of *N* HCl added	(*e*) Total vol.	(*f*) [HCl] = *d*/*e*	(*g*) γ corresponding with *f*	(*i*) [HCl free] = [H˙]/γ	(*j*) [HCl combined] = *f* − *i*	(*k*) cc. of combined HCl, 1·0*N* = *j* × *e*	(*l*) Equivs. of combined HCl	(*m*) log α/(1 − α)	(*n*) p_k
868·0	10·52	—	0·000	—	—	—	—	—	—	0·028	—	—
852·0	10·25	—	0·020	—	—	—	—	—	—	0·060	—	—
837·0	10·00	—	0·048	—	—	—	—	—	—	0·108	−0·91	9·09
826·0	9·82	—	0·080	—	—	—	—	—	—	0·162	−0·71	9·11
814·0	9·62	—	0·126	—	—	—	—	—	—	0·240	−0·50	9·12
804·0	9·44	—	0·170	—	—	—	—	—	—	0·314	−0·34	9·10
794·0	9·27	—	0·226	—	—	—	—	—	—	0·406	−0·17	9·10
784·0	9·11	—	0·280	—	—	—	—	—	—	0·498	+0·00	9·11
772·5	8·91	—	0·345	—	—	—	—	—	—	0·590	+0·16	9·07
761·5	8·73	—	0·402	—	—	—	—	—	—	0·708	+0·38	9·11
749·5	8·59	—	0·454	—	—	—	—	—	—	0·790	+0·57	9·16
734·0	8·26	—	0·506	—	—	—	—	—	—	0·876	+0·84	9·10
725·5	8·12	—	0·526	—	—	—	—	—	—	0·916	+1·04	9·16
708·0	7·83	—	0·550	—	—	—	—	—	—	0·950	+1·28	9·11
670·0	7·18	—	0·574	—	—	—	—	—	—	0·990	—	—
620·0	6·33	—	0·582	—	—	—	—	—	—	1·006	—	—
475·5	3·89	0·00013	0·600	5·60	0·107	0·74	0·00017	0·107	0·599	0·032	—	—
448·5	3·43	0·00037	0·620	5·62	0·110	0·74	0·0005	0·109	0·615	0·052	—	—
427·0	3·07	0·00085	0·656	5·66	0·116	0·74	0·0012	0·115	0·651	0·118	−0·87	2·20
414·5	2·85	0·0014	0·695	5·70	0·122	0·73	0·0019	0·120	0·684	0·175	−0·67	2·18
402·0	2·64	0·0023	0·748	5·75	0·130	0·73	0·0032	0·127	0·730	0·256	−0·46	2·18
392·0	2·47	0·0034	0·800	5·80	0·138	0·73	0·0047	0·133	0·771	0·320	−0·33	2·14
380·5	2·28	0·0053	0·868	5·87	0·150	0·72	0·0074	0·143	0·839	0·440	−0·10	2·18
369·5	2·09	0·0081	0·950	5·95	0·160	0·72	0·0113	0·149	0·886	0·514	—	—
358·0	1·90	0·013	1·048	6·05	0·174	0·71	0·0183	0·156	0·944	0·622	—	—
347·0	1·72	0·019	1·155	6·16	0·188	0·71	0·0268	0·161	0·992	0·690	—	—

Table III. *Titration of arginine with NaOH at 17°.*

0·1050 g. dissolved in 5·0 cc. of CO_2-free water and titrated with 1·08 *N* NaOH.

(*a*) E.M.F.	(*b*) p_H	(*c*) [H˙]	(*d*) cc. of 1·08 *N* NaOH added	(*d*) Expressed as *N*/1 NaOH	(*e*) Total vol.	(*f*) [NaOH] = *d*/*e*	(*g*) γ corresponding with *f*	(*h*) [OH′] = K_w/[H˙]	(*i*) [Free NaOH] = [OH′]/γ	(*j*) [Combined NaOH] = *f*/*i*	(*k*) cc. of combined NaOH, 1·0 *N* = *j* × *e*	(*l*) Equivs. of combined NaOH	(*m*) log α/(1 − α)	(*n*) p_k
904·0	11·28	$0·53 \times 10^{-11}$	0·021	0·023	5·02	0·0045	0·92	0·0015	0·0016	0·0029	0·0014	—	—	—
933·0	11·80	$0·16 \times 10^{-11}$	0·042	0·045	5·04	0·0090	0·92	0·0049	0·0054	0·0037	0·0018	—	—	—
950·0	12·10	$0·80 \times 10^{-12}$	0·070	0·076	5·07	0·0149	0·88	0·0099	0·0112	0·0037	0·0018	—	—	—
960·5	12·27	$0·54 \times 10^{-12}$	0·100	0·108	5·10	0·0212	0·88	0·0146	0·0166	0·0046	0·0024	—	—	—

Table IV. *Titration of arginine with HCl at 17°.*

0·1050 g. dissolved in 5·0 cc. of CO_2-free water and titrated with 1·0 *N* HCl.

(*a*) E.M.F.	(*b*) p_H	(*c*) [H$^{\cdot}$]	(*d*) cc. of *N* HCl added	(*e*) Total vol.	(*f*) [HCl] = *d*/*e*	(*g*) γ corresponding with *f*	(*i*) [HCl free] = [H$^{\cdot}$]/γ	(*j*) [HCl combined] = *f* − *i*	(*k*) cc. of combined HCl, 1·0 *N* = *j* × *e*	(*l*) Equivs. of combined HCl	(*m*) log α/(1 − α)	(*n*) p_k
867·5	10·70	—	0·000	—	—	—	—	—	—	0·028	—	—
849·0	10·35	—	0·022	—	—	—	—	—	—	0·064	—	—
839·5	10·21	—	0·042	—	—	—	—	—	—	0·098	−0·96	9·25
829·5	10·04	—	0·068	—	—	—	—	—	—	0·142	−0·78	9·26
820·5	9·88	—	0·100	—	—	—	—	—	—	0·196	−0·61	9·27
811·0	9·72	—	0·140		—	—	—	—	—	0·264	−0·44	9·28
802·5	9·56	—	0·184	—	—	—	—	—	—	0·338	−0·29	9·27
793·0	9·43	—	0·238	—	—	—	—	—	—	0·430	−0·12	9·31
784·0	9·27	—	0·288	—	—	—	—	—	—	0·514	+0·02	9·29
773·0	9·09	—	0·350	—	—	—	—	—	—	0·618	+0·20	9·29
763·0	8·93	—	0·402	—	—	—	—	—	—	0·708	+0·38	9·31
752·0	8·75	—	0·450	—	—	—	—	—	—	0·788	+0·56	9·31
736·0	8·47	—	0·498	—	—	—	—	—	—	0·870	+0·83	9·30
726·0	8·30	—	0·522	—	—	—	—	—	—	0·910	+1·00	9·30
705·5	7·94	—	0·548	—	—	—	—	—	—	0·956	—	—
646·0	6·90	—	0·570	—	—	—	—	—	—	0·990	—	—
465·0	3·75	—	0·590	—	—	—	—	—	—	0·026	—	—
444·0	3·38	0·0004	0·608	5·61	0·1085	0·74	0·0006	0·1080	0·606	0·046	—	—
431·0	3·16	0·0007	0·632	5·63	0·1122	0·74	0·0009	0·1113	0·626	0·096	−0·97	2·19
409·5	2·78	0·0016	0·700	5·70	0·1228	0·73	0·0023	0·1205	0·686	0·198	−0·61	2·17
398·0	2·58	0·0026	0·750	5·75	0·1305	0·73	0·0036	0·1269	0·729	0·270	−0·43	2·15
390·0	2·44	0·0036	0·800	5·80	0·1380	0·73	0·0049	0·1331	0·772	0·344	−0·28	2·16
380·0	2·26	0·0055	0·868	5·87	0·1480	0·72	0·0076	0·1404	0·824	0·434	−0·12	2·14
370·5	2·08	0·0083	0·948	5·948	0·1595	0·72	0·0155	0·1440	0·858	0·490	—	—
360·5	1·92	0·0120	1·028	6·028	0·1705	0·71	0·0169	0·1536	0·912	0·584	—	—
550·0	1·74	0·0182	1·130	6·130	0·1844	0·71	0·0263	0·1581	0·976	0·680	—	—
344·5	1·64	0·0229	1·200	6·200	0·1935	0·70	0·0327	0·1608	0·996	0·726	—	—

2. *Formaldehyde titrations.*

Titration curves were also determined in the presence of formaldehyde (Fig. 1), for this technique enables one to determine which of the p_K values relate to the amino- and which to the carboxyl groups and hence in any given case to obtain direct evidence [Harris, 1928, 1929] for or against the zwitterion formula—for which only indirect evidence had previously been available. The method, already applied by Harris to certain other amino-acids, rests on the following considerations. When formaldehyde is added to a simple base, the production of any methylene derivative, in equilibrium with the base, results in a diminution of the apparent basic strength, which is shown by a fall in the p_K value of the titration curve. Titration curves of acids on the other hand are, in general, naturally not affected by formaldehyde. Now when we titrate amino-acids, peptides or the like in formaldehyde we find that we get a number of p_K values shifted (towards diminished basicity) equal to the number of amino-groups in the molecule; and virtually no shift occurs in those remaining p_K values corresponding with the carboxyl groups. For example, glutamic acid, or reduced glutathione [Pirie and Pinhey, 1929], show two stationary and one shifted p_K values, lysine one stationary and two shifted, glycine one stationary and one shifted, and so on. The shifted p_K values in the amino-acid must clearly relate to the amino-groups for the following reasons. (1) In general, with mixtures of simple acids and bases of known p_K values it is always the basic and not the acid groups which are so shifted. (2) In ampholytes again, as just indicated, the number of p_K values shifted is equal to the number of amino-groups in the molecule and the number not shifted to the carboxyls. (3) If the contrary view were taken (*i.e.* that the shifted curve related to a carboxyl group and the unchanged to an amino-group) it would mean that formaldehyde causes no change in the measurable real basic strength, a conclusion difficult to reconcile with the fact that the formaldehyde is added (in Sørensen titrations) with the express purpose of weakening this basicity; it would also mean that there is an immense (*e.g.* 100,000 fold) increase in its *true* acid strength (*i.e.* in K_A, as opposed to k_a, the constant for an apparent acid group, as used in Harris's early papers where this effect of formaldehyde was first described),—a ridiculous supposition, most strikingly so in the case of molecules like polypeptides where the distance of the amino- from the carboxyl group would preclude the possibility that any slight change in the former would appreciably influence the strength of the latter. (4) Lastly it may be added that in its formaldehyde titration curve glycine behaves in a manner exactly parallel with ammonium acetate (the analogue of the zwitterion or "internal ammonium salt") and just contrary to a weak acid-weak base mixture (the analogue of the classical amino-acid formula).

In the case of arginine the p_K at 9·1 is due to an amino-group and that at 2·2 to a carboxyl group, for we found that the former p_K was shifted by formaldehyde in the direction of diminished basicity and the latter remained virtually

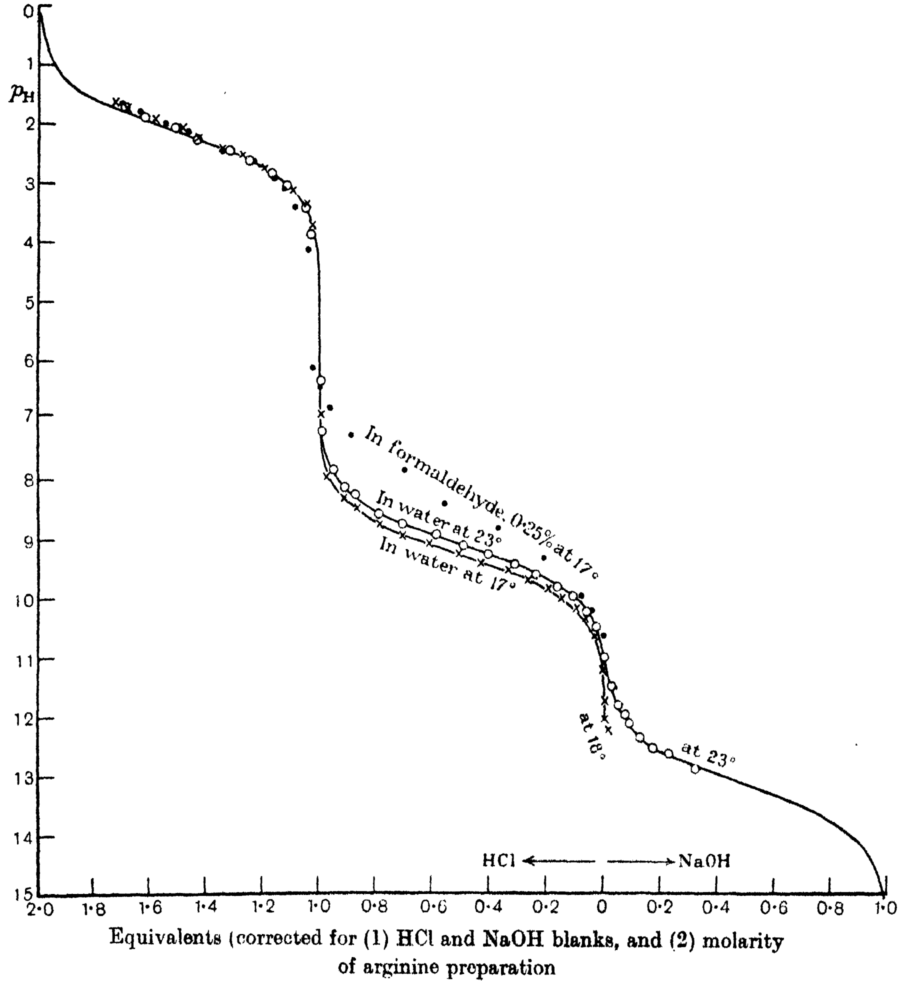

Fig. 1. Acid-base combination curve of arginine.

○ Experimental at 23° in water. × Experimental at 17° in water.

— Theoretical curve drawn from $p_{k_1}=2{\cdot}18$, $p_{k_2}=9{\cdot}09$, $p_{k_3}=13{\cdot}2$ at 23°; or $p_{k_1}=2{\cdot}17$, $p_{k_2}=9{\cdot}29$, $p_{k_3}>13{\cdot}2$, at 17°. (The temperature shift in p_{k_2} corresponds with the change in the value of p_{K_w} and is assigned to a basic (amino) group with a small temperature coefficient for its K_B, while p_{k_1} is assigned to the carboxyl group with a K_A having similarly a relatively small temperature coefficient.

• • • Titration in formaldehyde (0·25 %) at 17°. With formaldehyde there is no appreciable shift in the p_{k_1} curve but considerable upward shift in the p_{k_2} curve (increasing with further additions of formaldehyde). It is therefore concluded that the latter relates to a basic (amino) group, the basicity of which is weakened in presence of formaldehyde; and the former to the carboxyl group, since it is found in general that the true acidity of carboxyl and similar groups is not appreciably affected by HCHO.

unchanged. The p_k values 9·1 and 2·2 are of the same order as those found for the amino- and carboxyl groups respectively in other α-amino-acids, and this of course is an additional argument in favour of our assignment. One concludes therefore that the p_k at 13–14 is due to the very strongly basic guanidine group, an assumption which is further confirmed by a direct titration curve which we have carried out on free guanidine base itself, from which we find that its dissociation constant is no less in magnitude but in fact somewhat larger even than that which we have assigned for the combined guanidine group of arginine.

3. *Magnitude of* p$_k$ *temperature shifts.*

We have obtained yet further evidence supporting the above assignment, along somewhat novel lines, as follows. When titrations with the hydrogen electrode are carried out at several slightly differing temperatures, that curve which relates to the carboxyl group in the amino-acid (*e.g.* in glycine) shows no appreciable shift; that is, the p_k value has a small temperature coefficient. But, on the other hand, the titration curve for the amino-group shows a considerable shift with temperature, the magnitude of the shift depending mainly on the comparatively large change in the value of p_{K_W}[1], which of course enters into the hydrogen ion equation for a base, but not an acid; hence the magnitude of the real basic constant, p_{K_B}, calculated by subtracting p_k (*i.e.* "$p_{K_{AB}}$") from the value for p_{K_W} at the temperature in question, is, like the real acid constant, little affected by small temperature changes.

In general, the combination curve of a basic group will show less change with temperature when plotted on the OH than on the H scale, while that of an acid group will show less change with temperature when plotted on the H than on the OH scale; provided always that the temperature coefficient for the p_{K_A} (or p_{K_B}) is small compared with that for p_{K_W}. The reverse would be true were the temperature coefficients greater than that for p_{K_W}.

$$\text{For a base} \quad p_H = p_{K_W} - p_{K_B} - \log \frac{a}{1-a} \qquad \ldots\ldots 1,$$

$$\text{or} \quad p_{OH} = p_{K_B} + \log \frac{a}{1-a} \qquad \ldots\ldots 2.$$

$$\text{For an acid} \quad p_H = p_{K_A} + \log \frac{a}{1-a} \qquad \ldots\ldots 1,$$

$$\text{or} \quad p_{OH} = p_{K_W} - p_{K_A} - \log \frac{a}{1-a} \qquad \ldots\ldots 2.$$

That is, p_{K_W} with its appreciable temperature coefficient is involved in Equation 1, but not 2 for bases, and in Equation 2 but not 1 for acids. These relations between titration curve temperature shifts and temperature coefficient of dissociation constants will be discussed in detail in a later paper.

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[1] For the purpose of the present treatment we are adopting the classical, pre-Brønsted formulation of base-acid equilibrium.

In the case of arginine the first HCl curve is shifted just as is the NaOH curve with glycine; but the second HCl curve is not appreciably shifted. We assume therefore that the first HCl curve (p_{k_2}) relates to a basic group. Its p_k value, if expressed as a p_{K_A} constant, would show a relatively large shift with temperature; whereas expressed as a p_{K_B} constant ($p_{K_B} = p_{K_W} - p_k$) it shows only a relatively negligible temperature shift. On the other hand, the value p_{k_1} expressed as an acid constant shows little change with temperature: we accordingly assume, in further confirmation of the earlier evidence given above that it relates to an acid group. In the case of p_{k_3} there is a significant increase with lowering of temperature, so that we have here again additional evidence for relating it to a basic group.

Table V. *Zwitterion constants for arginine.*

	Temp.	p_k	p_{K_A}	p_{K_B} ($=p_{K_W} - p_k$)
p_{k_1}	17°	2·17	2·17	—
	23°	2·18	2·18	—
p_{k_2}	17°	9·29	—	4·88 (= 14·17 − 9·29)
	23°	9·09	—	4·87 (= 13·96 − 9·09)

Our conclusions may be summarised and compared with Simms's, as follows.

Table VI. p$_k$ *values*[1] *of arginine.*

	Birch and Harris concentration = 0·1 N		Simms
	temp. = 23°	temp. = 17°	
Guandine group	13·2	> 13·2	8·15
α-Amino-group	9·09	9·29	9·64
Carboxyl group	2·18	2·17	2·29

II. Histidine.

Similar methods were applied to histidine, and details and results are appended below.

It will be noticed that the titration curve includes a second group titrating with acid at p_k 1·8, not shown by Hirsch.

Since this work was concluded Schmidt *et al.* [1929] have published values for histidine which are in excellent agreement with our figures, although these authors express the NaOH and second HCl curves as k_a and k_b constants respectively while we have definitely established them as K_B and K_A constants (from formaldehyde titrations, and a comparison of the magnitude of the temperature shift; see Fig. 2). p_{k_2} (first HCl curve) remains a basic group, and no doubt relates to the iminazole group.

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[1] k is used throughout for the apparent or titration constant, and is not necessarily exactly identical with the true dissociation constant of the formal classical definition. K_A and K_B denote the titration constants expressed in the zwitterion mode, or the constants of true acids and bases: k_a and k_b denote the constants of apparent acids and bases.

Table VII. *Titration of histidine monohydrochloride hydrate with NaOH at 23°.*

Hofmann la Roche specimen: 0·1047 g. dissolved in 5 cc. of CO_2-free water, to make decimolar solution, and titrated with 1·08 *N* NaOH.

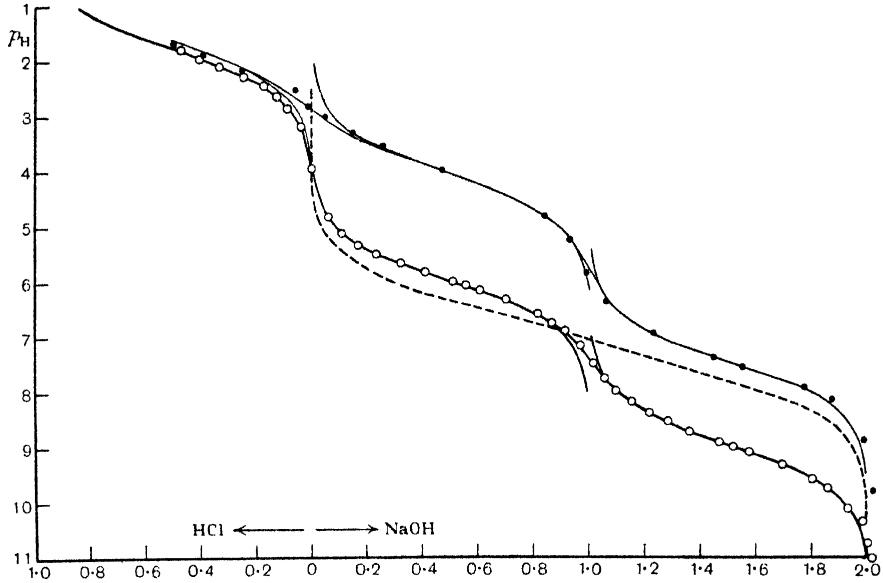
E.M.F.	p_{H}	cc. of NaOH, 1·08 *N*	NaOH in equivalents	log $a/(1-a)$	p_{k}
477·0	3·94	0·000	0·000	0·00	—
527·5	4·80	0·028	0·060	+1·19	5·99
545·0	5·10	0·048	0·104	+0·93	6·03
557·5	5·31	0·076	0·164	+0·71	6·02
568·0	5·49	0·108	0·234	+0·51	6·00
577·0	5·64	0·148	0·320	+0·33	5·97
587·0	5·81	0·190	0·410	+0·16	5·97
597·0	5·98	0·234	0·506	−0·01	5·97
600·0	6·03	0·256	0·554	−0·09	5·94
605·0	6·12	0·278	0·602	−0·18	5·94
616·0	6·30	0·324	0·700	−0·37	5·93
632·5	6·58	0·376	0·812	−0·63	5·95
642·0	6·75	0·400	0·864	—	—
649·0	6·88	0·422	0·912	—	—
666·5	7·16	0·447	0·966	—	—
685·0	7·48	0·470	0·016	—	—
700·0	7·74	0·488	0·058	+1·21	8·95
713·5	7·97	0·506	0·094	+0·98	8·95
725·5	8·17	0·532	0·150	+0·75	8·92
737·5	8·38	0·562	0·216	+0·56	8·94
746·0	8·52	0·592	0·280	+0·41	8·93
757·0	8·71	0·630	0·360	+0·25	8·96
768·5	8·91	0·678	0·464	+0·06	8·97
774·5	9·01	0·700	0·512	−0·02	8·99
780·0	9·11	0·728	0·572	−0·12	8·99
794·0	9·34	0·782	0·690	−0·35	8·99
809·0	9·60	0·832	0·798	−0·59	9·01
818·0	9·75	0·860	0·858	−0·77	8·98
835·0	10·12	0·892	0·926	—	—
853·0	10·34	0·918	0·980*	—	—
877·0	10·75	0·932	1·002*	—	—
894·0	11·04	0·944	1·026*	—	—

* Corrected for blank.

Table VIII. *Titration of histidine monohydrochloride hydrate with HCl at 23°.*

Hoffmann la Roche specimen: 0·1047 g. dissolved in 5 cc. of CO_2-free water, to make decimolar solution, and titrated with 1·0 *N* HCl.

(*a*) E.M.F.	(*b*) p_{H}	(*c*) $[\mathrm{H}^{\cdot}]$	(*d*) cc. of *N* HCl added	(*e*) Total vol.	(*f*) $[\mathrm{HCl}]=d/e+0{\cdot}1$	(*g*) γ corresponding with *f*	(*i*) [HCl free] $=[\mathrm{H}^{\cdot}]/\gamma$	(*j*) [HCl combined] $=f-i$	(*k*) cc. of combined HCl, $1{\cdot}0N=j\times e$	(*l*) Equivs. of combined HCl	(*m*) log $a/(1-a)$	(*n*) p_{k}
477·0	3·96	0·0001	0·000	5·00	0·1000	0·74	—	0·1000	0·500	0·000	—	—
431·0	3·17	0·0007	0·024	5·02	0·1044	0·74	0·0009	0·1035	0·520	0·040	−1·38	1·79
413·0	2·86	0·0014	0·048	5·05	0·1095	0·74	0·0019	0·1077	0·544	0·088	−1·02	1·84
399·0	2·63	0·0023	0·078	5·08	0·1138	0·74	0·0031	0·1107	0·564	0·128	−0·83	1·80
387·5	2·43	0·0040	0·116	5·12	0·1203	0·73	0·0055	0·1148	0·588	0·176	−0·67	1·76
378·0	2·27	0·0054	0·162	5·16	0·1284	0·73	0·0074	0·1210	0·624	0·248	−0·48	1·79
368·0	2·09	0·0081	0·224	5·22	0·1392	0·73	0·0111	0·1281	0·669	0·338	−0·29	1·80
358·5	1·93	0·012	0·290	5·29	0·1495	0·72	0·0167	0·1328	0·703	0·406	−0·17	1·76
350·0	1·79	0·016	0·358	5·36	0·1601	0·72	0·0222	0·1379	0·739	0·478	−0·04	1·75



Equivalents (corrected for HCl and NaOH blanks) added to histidine monohydrochloride hydrate

Fig. 2. Acid-base combination curve of histidine.

○ Experimental (23°).

—— Theoretical curve for p_k values at 1·78, 5·97, 8·97. At a lower temperature, viz. 17°, p_{k_2} and p_{k_3} values were notably increased and therefore are assigned to basic groups. By analogy with all the α-amino-acids, the p_k at about 9—*i.e.* p_{k_3}—no doubt relates to the α-amino-group and hence the p_k at 6—*i.e.* p_{k_2}—relates to the iminazole group. Formaldehyde titration, again, confirms the assignment of p_{k_1} to the carboxyl and p_{k_2} and p_{k_3} to basic groups.

••• Formaldehyde titration readings, with theoretical p_k curves.

– – – Curve given by Hirsch showing only one acid and one basic group each possessing different p_k values from the above, and with no point of inflection at about p_H 7·5.

Table IX. p_k *values for histidine.*

Temp.	Calculated by Harris [1923, 1] from Kanitz's conductivity measurements	Hirsch [1924]	Simms [1928]	Schmidt, Appleman and Kirk [1929]	Birch and Harris
	25°	18°	25°?	25°	23°
p_{k_1}	1·7	—	1·46	1·82	1·78
p_{k_2}	5·8	6·32	6·06	6·01	5·97
p_{k_3}	8·7	7·74	9·41	9·17	8·97

SUMMARY.

1. The p_k value 8·15 given by Simms for the guanidine group in arginine, on the basis of which he postulated the existence of a "prearginine" group in proteins, is widely divergent from a value, viz. $p_k = ca.$ 13 to 14, previously deduced (1) by one of us from titration readings and Kanitz's conductivity data, and (2) electrometrically by Hunter and Borsook.

An accurate redetermination has given us the three p_{K} constants, 13, 9·09 and 2·18 ($t = 23°$, 0·1 N), in good agreement with earlier values.

It is shown, by Harris's formaldehyde titration technique, and by a new method involving measurement of the shift of $p_{\mathrm{K}_{\mathrm{AB}}}$ with temperature, that the first of these relates to the guanidine group, the second to an amino-group and the third to the carboxyl group. That is, the magnitude of the guanidine group's dissociation constant as determined by us is 100,000 times greater than that given by Simms.

2. Since the titration curve of histidine was originally reported by one of us to consist of three p_{K} components, whilst Hirsch described two only, a redetermination has been made which gives the three p_{K} values 8·95, 5·98 and 1·78 (at 23° and 0·1 N); the first relates to the amino-, the second to the iminazole and the third to the carboxyl group.

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OBITUARY NOTICES.

THORBURN BRAILSFORD ROBERTSON (1884–1930).

THORBURN BRAILSFORD ROBERTSON, Professor of Biochemistry and General Physiology at the University of Adelaide, and Director of the Animal Nutrition Laboratory of the Commonwealth Council for Scientific and Industrial Research, died from pneumonia at Glenelg, South Australia, on the 18th January, 1930.

Biological science has lost, in Robertson, one of its most brilliant and energetic exponents. It is impossible, in a short note, to discuss the many problems which at different times absorbed his interest. He was one of those vigorous, fearless thinkers who seem to be entirely free from the inhibitions which restrain most of us in narrow paths of scientific enquiry.

His magnificent courage and imagination led him into every aspect of biology which presented problems to his consciousness. Perhaps he attempted more than he could possibly expect to accomplish. His industry was prodigious and his output was remarkable, for he suffered ill health throughout his life and died at the comparatively early age of 46.

His work in recent years has been mainly concerned with the general problem of growth, but he had unusually broad interests and his publications covered a great variety of subjects. The first investigation on which he worked intensively was the physical chemistry of the proteins. He was a pioneer in this field and was one of the first workers to show that protein chemistry was amenable to quantitative treatment. The value of his early experimental work on the binding power of proteins for bases has been recognised on all sides, though his theoretical conceptions on the nature of protein salts failed to gain adherents. The problem of growth in all its aspects was his next chief interest, but he never suffered mental confinement in any one compartment of science. He went without hesitation into all phases of general physiology. At the time of his death he was working hard at animal nutrition, as Director of the Waite Research Institute at Adelaide, and his contributions to the biochemical aspects of sheep farming in Australia promise to be of considerable economic significance for the Dominion.

He was a fluent, lucid and inspiring writer. His textbook of Biochemistry is unique in the success with which the subject matter is conveyed to the reader through the personality of its author. This book was written in two or three months with practically no reference to notes or books, and differs as widely as possible from the usual compilations which serve the purpose of textbooks. It is not only one of the most readable, but also one of the most stimulating works on the subject.

Among his voluminous scientific writings are many which deal with the social relations of science, and no pen has more eloquently expressed the claims of the history of science to a place among the humanities. He once wrote a fascinating description of human biochemistry for little children under the intriguing title of the *Universe and the Mayonnaise.*

Robertson had a most attractive personality, and was beloved by his colleagues in California, Toronto and Adelaide. Like all strong characters he made enemies, but never among his close associates. His originality of mind made him a stimulating colleague, and to his students he was as gracious as he was inspiring.

His enthusiasm was communicated to those around him, and his return to Adelaide in 1919 to succeed Sir Edward Stirling in the Chair of Physiology was followed by an expansion of interest in scientific research, not only in Adelaide but throughout the Dominion. Robertson founded the *Australian Journal of Experimental Biology and Medical Science,* to which he was a frequent contributor.

Robertson's main service to science was in the realm of ideas. His practical work was of lesser significance, but in almost every direction in which his imagination led him he either contributed new conceptions or reoriented prevailing ideas.

Biochemistry at this period has an abundance of practical workers, but a dearth of vision. Men of Robertson's rare quality can ill be spared.

H. W.

ROBERT LAUDER MACKENZIE WALLIS (1886–1929).

MACKENZIE WALLIS was a Cambridge man, a scholar of Downing College, with a first class in the Natural Science Tripos, Part I. His first appointment was that of Lecturer in Chemical Physiology at Cardiff and his second that of Demonstrator in Chemical Pathology at St Bartholomew's Hospital. Soon after the war began Mackenzie Wallis went to India with the rank of Captain, where he remained to the end of the war. On his return in 1919 he was appointed Lecturer in Chemical Pathology at St Bartholomew's in succession to Sir A. E. Garrod, and in the same year he was awarded the M.D. degree at Cambridge for a thesis which gained him the Horton-Smith Prize. In 1921 he was appointed Chemical Pathologist to the Hospital.

Mackenzie Wallis was a hard worker; he organised the new and rapidly growing department of Chemical Pathology at St Bartholomew's and carried out a great mass of routine work in this subject himself, and supervised the work of others. The experience he gained in this way was placed at the service of his colleagues and of many others who were glad to avail themselves of it. He was constantly engaged on research in Chemical Pathology and the results of this work will be found in various clinical journals. Mackenzie Wallis was a genial and charming man, always ready to impart his knowledge to others and as ready to learn from others: his death on April 1st, 1929, at the age of 43 years, was a severe blow to his many friends at St Bartholomew's Hospital and elsewhere.

W. H. H.

LXIV. THE REDUCTION OF METHYLENE BLUE IN MILK.

THE INFLUENCE OF LIGHT.

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Three types of reaction which will bring about the reduction of methylene blue added to milk have been known for a considerable time and have been utilised in various ways.

(i) Schardinger [1902] has shown that milk contains an enzyme which is able to catalyse the reduction of methylene blue in the presence of an aldehyde. Trommsdorff [1909] has proved that the enzyme is a constituent of the milk itself, and is independent of micro-organisms. Other observers have shown that aldehydes may be replaced by xanthine, and methylene blue by nitrates. Hence the reaction is now recognised as a typical oxidation-reduction in which hydrogen donator and hydrogen acceptor are enabled to interact through the presence of an enzyme or catalyst.

(ii) Milk which has been heated for a short time to a temperature over 100° will bring about the decoloration of methylene blue. The mechanism of this reaction has not yet been definitely established. The possibility of enzyme action is of course excluded. Barthel [1925] considers that an oxidation-reduction occurs in which citrate acts as hydrogen donator, methylene blue as hydrogen acceptor, and some inorganic constituent of milk as catalyst. Schwartz [1929], however, has brought forward evidence in favour of the theory that hydrolytic products of the milk proteins, probably those containing sulphydryl groups, are the reducing agents.

(iii) Milk containing living bacteria will decolorise methylene blue. The reaction in this instance is caused by an enzyme associated with the micro-organisms, and most probably is an oxidation-reduction in which the methylene blue acts as a hydrogen acceptor. The corresponding oxidation, which occurs simultaneously, must be a reaction which supplies energy for the growth of the organisms. Clark, Cohen, and Gibbs [1928] have shown that a reaction takes place in the absence of methylene blue, and that the dye, if present only in small quantity, is merely an indicator of reducing potential, analogous to an indicator of hydrogen ion concentration. Milk has therefore no specific function in the reaction, but is merely the source of substances suitable for the supply of energy to bacteria.

The third reaction has been used for many years as a means of determining the degree of contamination of market milk supplies, and it was while applying it for this purpose that the observation was made that light has a significant effect on the progress of the decoloration of methylene blue in milk. Some samples of milk to which methylene blue had been added were incubated in a water-bath with glass sides, designed to facilitate observation of the tubes. All the samples gave unexpectedly short reduction times averaging 2 to 3 hours. On repeating the test with the same samples of milk, but using an ordinary metal water-bath, many of the samples took more than 7 hours to reduce. Sunlight evidently had influenced the progress of the decoloration.

So far as can be ascertained, there is no reference in the literature on methylene blue reduction in milk to any observation of the effect of light. Clark, Cohen and Gibbs [1928] have observed an oxidising effect of ultra-violet light on methylene white in aqueous solution, and Webster, Hill and Eidinow [1924] have used the decoloration of methylene blue in the presence of acetone for measuring the intensity of ultra-violet light. The latter reaction is said to be due to the decomposition of the acetone with the formation of reducing substances.

Experiments were devised in an attempt to find an explanation of the mechanism of the process in milk. It was necessary first to determine whether an enzyme of the milk or of the contaminating organisms was concerned in the reaction. Reductase tests were set up in duplicate with fresh milk and some of the same milk heated for half an hour in a boiling water-bath. One series was incubated in water at 37° in a thin-walled glass vessel exposed to bright sunlight, and the other series was incubated in darkness. The tubes which were not exposed to sunlight retained the blue colour for more than 7 hours. In the series exposed to sunlight there was very little difference between the heated and unheated milk samples. They showed decoloration in times which varied from half an hour to several hours, according to the intensity of the sunlight. Usually the heated milk samples were decolorised in rather shorter time than the corresponding unheated samples, but this was not always the case, and probably the difference was due to the loss of dissolved oxygen brought about by heating, and the varying degree of subsequent aeration. In every instance the lower part of the tube was the first to lose the blue colour and the decoloration progressed upwards. There was always a blue ring left at the top of the tube and on dull days the decoloration was never complete in the upper half of the milk. In particularly intense sunlight the decoloration proceeded until there was merely a blue ring at the milk surface. Thereafter the blue colour was slowly regenerated in the upper part of the tube, and sometimes reached halfway down the milk column after several hours. In some experiments a layer of liquid paraffin was superimposed on the milk to avoid any influence of atmospheric oxygen, but with heated milk no difference was observed. In unheated milk decoloration occurred a little more rapidly when the milk was covered with paraffin, but the differences were hardly significant. This is in accord with the findings of Thornton and Hastings [1929] that diffusion of atmospheric oxygen into milk is much too slow to affect the reductase test. The number of bacteria in the milk seemed to have no bearing on the phenomenon in unheated milk, so long as the reduction caused by the organisms was sufficiently delayed, so that it was not superimposed on the light effect. In some experiments milk was obtained with sterile precautions directly from the cow into a sterile flask and the test was performed immediately. The results were exactly the same as those obtained with milk several hours old. This rules out the possibility that some heat-stable substance produced by bacterial action is necessary for the reaction.

It is evident therefore that the reaction takes place between some chemical constituent of the milk and methylene blue under the influence of sunlight, or that some reaction takes place in the milk itself which produces a reducing potential sufficient to reduce methylene blue. In the latter case the dye would be acting merely as an indicator of the oxidation-reduction potential; in the former case it would be essential to the progress of the reaction. Electrometric measurements of the oxidation-reduction potential in the presence and the absence of the dye will be necessary to decide which explanation is correct.

In a consideration of the substances in milk which may be concerned in the reaction, the milk-fat attracted attention, for it is well known that some of the unsaturated fats in cream undergo oxidation more readily in the presence of light. To determine whether the fat was responsible for the reducing action, milk was run through a centrifugal separator and reductase tests were performed on the original milk and on the separated milk. In bright sunlight the tubes containing separated milk suffered no decoloration, whereas the whole milk tubes became colourless as usual in a longer or shorter time, depending on the intensity of the light. The blue sometimes paled temporarily in separated milk, but returned in an hour or two to its full depth. A layer of liquid paraffin on the milk again had no effect, so that the greater ease of diffusion of oxygen into separated milk could not be the explanation of the absence of decoloration. The substance necessary for the reaction must be a constituent of the cream.

As a further test of the fat oxidation hypothesis some experiments were carried out in which salts of known fatty acids were added to separated milk in an endeavour to replace the substance which reacts with methylene blue. The sodium salts of palmitic and oleic acids were chosen as representatives of the saturated and unsaturated types. Clear-cut differences were immediately observed in reductase tests incorporating these substances and separated milk. The sodium palmitate had no effect within 7 hours, while the sodium oleate induced reduction in a short time in sunlight and had no effect in darkness. This seems strong evidence in favour of the theory that the reduction of methylene blue in milk under the influence of sunlight is an oxidation-reduction phenomenon in which unsaturated fats are oxidised and methylene blue acts as hydrogen acceptor. The occasional return of colour in strong sunlight in the top layers of milk where fat is collecting may possibly be due to an autoxidation of the oxidised fats resulting in a final oxidation-reduction potential sufficient partly to re-oxidise the methylene blue. Further evidence would be needed on this point.

Further work is projected in which the phenomenon will be examined in a more quantitative manner with the aid of a quartz mercury-vapour lamp, for it seems most probable that the active agent is ultra-violet light and that more powerful effects will be observed than with sunlight acting through one or two layers of glass. The phenomenon has obvious practical importance in connection with the keeping qualities of butter-fat in the presence of hydrogen acceptors.

EXPERIMENTAL.

In all reductase tests 20 cc. of milk were measured into a 6″ by ⅝″ test-tube and 1 cc. of methylene blue solution was added. The dye solution was prepared from tablets supplied by Blauenfeldt and Tvede, Copenhagen, for the purpose of determining the bacterial reduction of methylene blue in market milk. The final concentration of dye in the milk was about 1 in 100,000. The tubes used had been previously sterilised and when the tubes were inverted to mix the contents, the operator's finger was washed with alcohol on each occasion. The samples were exposed to sunlight by immersing them in water at 37° in a thin beaker set close to the laboratory window.

The effect of sunlight on methylene blue reduction in heated and unheated whole milk.

Milk was measured into test-tubes some of which were immersed in a boiling water-bath for half an hour. The heated samples were thereafter cooled in water and inverted several times to aerate the milk to a moderate degree. Methylene blue was added to all the tubes and one set was incubated exposed to sunlight while a control set was incubated in darkness.

Table I. *Times of decoloration in hours.*

	In the light		*In the dark*	
	Unheated milk	*Heated milk*	Unheated milk	Heated milk
Bright day	*2·25*	*1·75*	All over 7	All over 7
Bright day	*1·5*	*0·5*	,,	,,
Dull day	1·5 (only ½ decol.)	1·5 (only ½ decol.)	,,	,,
Dull day	4 (only ½ decol.)	4 (only ½ decol.)	,,	,,
Bright day	1·5	1·75	,,	,,

The above results are typical of many observations. On dull days the decoloration usually progressed no farther when the lower half of the milk column was colourless. The variation between unheated and heated milks is probably accounted for by different degrees of aeration. The time of decoloration may also vary with different milks.

The effect of sunlight on methylene blue reduction in whole and separated milk.

Milk was run through a sterilised centrifugal separator and both the original milk and the separated milk were used for reductase tests. Both milks were used in the heated and unheated states and in some tubes a layer of liquid paraffin was added. Bacteriological tests on the milk before and after separation showed that no measurable contamination had taken place.

Table II. *Times of decoloration in hours, in the light.*

	Whole milk	Whole milk + paraffin	Whole milk heated	Whole milk heated + paraffin	Separated milk	Separated milk + paraffin	Separated milk heated	Separated milk heated + paraffin
Bright day	2	0·5	0·5	0·5	>7	>7	>7	>7
Dull day	1·5 (only ½ decol.)	2 (only ½ decol.)	1·5 (only ½ decol.)	3·5 (only ½ decol.)	>7	>7	>7	>7
Dull day	4 (only ½ decol.)	4 (only ¾ decol.)	4 (only ½ decol.)	4 (only ⅓ decol.)	>7	>7	>7	>7
Bright day	1	—	—	—	>7	—	—	—
Bright day	1·5	—	—	—	>7	—	—	—

All the controls incubated in darkness were not decolorised in 7 hours. The difference between whole milk and separated milk was always clear cut and it is evident that diffusion of atmospheric oxygen has no appreciable effect.

The effect of the addition of salts of fatty acids to separated milk.

Milk was freed from fat as previously described. Sodium oleate and sodium palmitate were added in amounts of approximately 0·5 g. to different tubes of whole and separated milk, and partially dissolved by gentle shaking. Reductase tests were set up as before.

Table III. *Times of decoloration in hours, in the light.*

	Whole milk	Separated milk	Whole milk + Na oleate	Whole milk + Na palmitate	Separated milk + Na oleate	Separated milk + Na palmitate
Bright day	1·75	>7	1	1·25	0·5	>7
Bright day	—	>7	—	—	1·5	>7

All the controls incubated in darkness were not decolorised in 7 hours. These are typical of several experiments on similar lines. There is thus strong evidence that an unsaturated acid such as oleic acid can replace some constituent of the cream in this reaction with methylene blue.

SUMMARY.

1. Methylene blue added to fresh milk of good quality is reduced in a short time in the presence of sunlight at 37°. In darkness at 37° no decoloration occurs within 7 hours.

2. The reaction in sunlight is not due to an enzyme, for it proceeds equally well in milk which has been heated to 100° for 30 minutes.

3. Milk from which the fat has been removed by centrifugal separation no longer gives the reaction, but the activity of the milk can be restored by an addition of sodium oleate. Sodium palmitate has not a similar action.

4. It is suggested that sunlight catalyses an oxidation-reduction reaction in which unsaturated fats are oxidised and methylene blue is reduced.

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LXV. THE TRACES OF COMBUSTIBLE GASES IN HUMAN EXPIRED AIR.

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The stimulus to the present investigation was furnished by the observations of Campbell [1929], who showed that the gases present in the peritoneal cavities of animals may contain as much hydrogen (produced by intestinal putrefaction) as corresponds to a tension of the order of 20 mm. Hg. Some of this hydrogen must dissolve in the blood and later diffuse through the lungs into the expired air. The object of the experiments herein recorded has been to determine the extent to which such a transfer of hydrogen or other combustible gas takes place in the human subject, in order to decide whether this constitutes a factor necessary to be taken into consideration in the calculation of the total respiratory exchange from observations carried out with an open circuit respiration chamber. In using such an apparatus the volume of the ingoing air is calculated from the percentage of nitrogen in the outgoing air, the total amount of nitrogen being assumed to be unaltered during its passage through the chamber. But any hydrogen given off from the subject would be returned as unabsorbable gas, *i.e.* nitrogen, in the analysis of the outgoing air and would influence the calculation of the volume of air entering the chamber and so of the respiratory exchange of the subject.

The possibility of carrying out such an investigation depended on the circumstance that there was available the gas analysis apparatus accurate to 0·001 % already described by Krogh [1920]. In using such an apparatus for the estimation of minute traces of combustible gases, two points of technique must be carefully observed. Firstly, the solubility of oxygen and nitrogen in the potash solution used for the CO_2 absorption becomes a factor of very significant magnitude: in order to avoid errors from this source, it is usual to keep this potash solution saturated with air at the temperature of the apparatus, but this restricts the use of the apparatus to the analysis of gas mixtures not differing very appreciably in composition from atmospheric air. Then, secondly, it has already been shown [Krogh, 1919] that pure atmospheric air itself shows a just measurable diminution in volume amounting to about 0·0005 cc. for each minute of exposure to the hot platinum wire of the combustion pipette—an effect that cannot be due to the presence of combustible gases in the atmosphere as it can be repeatedly obtained with the same sample of air. This means that there will never be an absolutely final end-point to a combustion analysis; when all the combustible gases present have been burned, a further slight diminution of volume will always be obtainable on continuing the combustion. The diminution of volume due to this effect in pure air becomes added to that due to the burning of any combustible gases that may be present. These considerations led to the adoption of the following technique.

In order to have the maximum amount of hydrogen for measurement we analysed not the expired air as directly collected but air that had been rebreathed until its hydrogen tension had come into equilibrium with that in the blood. A simple closed circuit was used consisting of a pair of respiratory valves, a tower of loosely packed soda-lime and a Douglas bag. Into the latter there were drawn through a cotton-wool filter about 5 l. of outside air and to this was added enough oxygen from a cylinder to cover the estimated needs of the subject during the experiment. In this way the composition of the air in the bag at the end of the rebreathing period did not differ greatly from that of the atmosphere, and solubility errors did not enter into the analysis. It was found convenient to observe the volume of air in the bag by attaching a sheet of metal to its upper surface and connecting this by a thread to a simple lever: in this way it was easy to see the point at which all the added oxygen had been used up by the subject, which was the point at which the rebreathing was stopped. To ensure that the results were not being affected by possible contaminating traces of combustible gases, both the air and the oxygen used were passed slowly through about 6 cm. of red-hot wire-form CuO before being collected in the bag. As a further precaution in each experiment a control sample of air was withdrawn from the bag for analysis before the oxygen was added. It was also proved that the rubber lining of the bag did not give off any appreciable amount of combustible gas to air stored within it. Lastly, in order to avoid any possible formation of hydrogen by the action of moisture on oxidisable metals, no metal parts other than the heavily nickel-plated valves were used in the respiration circuit. Every effort was made to carry out the analyses as uniformly as possible. In all cases after a preliminary absorption of any CO_2 present the gas was passed to and fro for 6 minutes (in three carefully measured periods of 2 minutes) over the platinum wire of the combustion pipette heated to dull redness. After measurement of the resulting contraction a second CO_2 absorption was carried out.

Table I contains the results of duplicate determinations made in this way on control samples of atmospheric air collected and treated as mentioned in each case. It will be seen that these data confirm the former statement that in pure air for each minute of exposure to the hot wire shrinkage occurs to the extent of about 0·001 % of the original volume, but that the volume of the potash-absorbable product apparently formed is always less than twice the contraction on combustion. This indicates that, whatever its explanation, the phenomenon cannot be due to the presence of traces of carbon monoxide in the air analysed and this is confirmed by the observation that the effect is equally well obtained with air that has been passed over red-hot copper oxide.

Table I. *Control combustions with atmospheric air.*

Date	Material analysed	×0·01 % of volume taken for analysis (50 cc.)	
		Contraction on 6 mins. combustion	Subsequently absorbed by KOH
3. v	Filtered outside air	0·50 0·35	0·85 0·80
7. v	Air after exposure to red-hot CuO	0·50 0·50	0·50 0·65
10. v	Filtered outside air	0·40 0·50	1·10 0·90
10. v	Air after storage in Douglas bag 6·5 hours	0·70 0·65	0·75 0·75
13. v	Air after storage in Douglas bag 3 days	0·80 0·65	1·00 1·00
15. v	Filtered laboratory air	0·55 0·25	0·65 0·55
19. v	Filtered outside air	0·50	0·55
1. vi	Filtered outside air	0·45	0·75
4. vi	Air from compressed air supply after exposure to red-hot CuO	0·50	0·85

Table II. *Analyses of rebreathed air.*

Date	Experiment and subject	×0·01 % of volume taken for analysis (50 cc.)			
		Contraction on 6 mins. combustion	Subsequently absorbed by KOH	H_2	CH_4
30. iv	Filtered air + 5 l. O_2. Rebreathed 15 mins. Subject P.	1·00 1·00	1·20 0·65	0·25	0·10
2. v	Filtered air + 5 l. O_2. Rebreathed 15 mins. Subject N.	1·20 1·50	1·35 1·40	(?)	0·55
15. v	Air + 5 l. O_2 passed over red-hot CuO. Rebreathed 15 mins. Subject R.	4·75 4·55	0·90 0·70	2·80	0·00
18. v	Air + 5 l. O_2 passed over red-hot CuO. Rebreathed 15 mins. Subject P.	1·90 2·00	1·20 1·00	0·25	0·55
31. v	Air + 6 l. O_2 passed over red-hot CuO. Rebreathed 15 mins. Subject R.	4·00 3·90	1·20 1·00	1·85	0·35
4. vi	7·5 l. air + 20 l. O_2 all passed over red-hot CuO. Rebreathed 63 mins. Subject P.	2·15 1·15 (?)	1·45 1·35	0·30	0·60

Turning now to the analyses of the rebreathed air recorded in Table II, it will be seen at once that the contractions on combustion and the subsequent amounts of absorption by potash are invariably larger than with atmospheric air, indicating that traces of combustible gases are in fact expired by the human subject. The subjects used for these observations were all examined in the absorptive period either in the middle of the morning (Exps. 1, 2 and 4) or after lunch (remaining experiments). That these traces of expired combustible gases do not consist of carbon monoxide derived from tobacco smoke is evident from the fact that the highest figures were obtained with the subject R. who is a non-smoker. Prolongation of the rebreathing period from

15 minutes to 63 minutes as in the last experiment recorded led to no significant increase in the amount of combustible gas collected.

In the last two columns of Table II the amounts of combustible gases in the rebreathed air have been calculated on the assumption that they consist of hydrogen and methane only. In making this calculation the contraction on combustion and subsequent absorption by potash obtained on the same day with a control sample of atmospheric air, collected as mentioned from the Douglas bag just before each experiment, were subtracted from the corresponding data obtained with the rebreathed air.

In order to determine approximately the rate at which these gases are given off from the body, let us take the highest percentage of combustible gas obtained, namely the 2·80 × 0·01 % from the experiment of 15. v. This appears to be made up entirely of hydrogen. This percentage corresponds to a tension in moist air at body temperature of approximately 0·2 mm. Hg. Taking this as the same as the tension of hydrogen in the mixed venous blood, and assuming an average rate of blood flow through the lungs of 5 l. per minute and a solubility of hydrogen in blood equal to that in water under the same conditions, the amount of hydrogen carried to the lungs per minute works out to be 0·02 cc. But during a respiratory experiment on a human subject about 60 l. of air pass through the chamber per minute, so that if the blood gave off the whole of its hydrogen while passing through the lungs this would add only 1 part of inert gas to 3×10^6 parts of outgoing air—a change in composition that is about one-thirtieth of the smallest detectable by the most accurate technique yet devised. We may conclude with certainty, therefore, that, whatever may be the exact nature of these traces of combustible gases in human expired air, even if they were produced at 10 times the maximum rate here observed, they would be quite without influence on the results of the most accurate respiratory experiments that have so far been made.

SUMMARY.

1. The slight shrinkage in volume when pure atmospheric air is submitted to combustion analysis is confirmed. This effect cannot be due to the presence of traces of ordinary combustible gases as it is obtained in air that has been passed over red-hot copper oxide.

2. Air that has been rebreathed by a human subject contains traces of combustible gases in amounts that indicate a rate of production of about 0·02 cc. per minute. This is far too small to be of significance in determinations of total respiratory exchange.

It is a great pleasure to the author to acknowledge here the stimulating help of Professor Krogh during the carrying out, at his suggestion, of this investigation.

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LXVI. THE INFLUENCE OF ARGININE UPON THE GROWTH RATE OF A TRANSPLANTABLE TUMOUR IN THE MOUSE.

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Research in nutrition has shown that the various amino-acids differ among themselves in respect of their relative importance in relation to growth in the immature animal, and to maintenance of weight in the adult [Mitchell and Hamilton, 1929].

The results of Ackroyd and Hopkins [1916] provided evidence that arginine and histidine are interconvertible in the body, and that the presence of one or the other is essential for the processes of growth; that arginine is necessary for the maintenance of weight had been demonstrated by Geiling [1917] in the mouse. Confirmation of the indispensability of histidine was obtained by Rose and Cox [1924], but they reported that arginine, as a dietary constituent, was without effect on weight; similar results were recorded by Rose and Cook [1925]. The results obtained by Stewart [1925] were in agreement with the latter in respect of histidine, but with regard to the significance of arginine they were intermediate between the results of Ackroyd and Hopkins, and those of Rose and Cox. From these more recent investigations it appeared that arginine and histidine were not mutually replaceable in metabolism, and that arginine was definitely inferior to histidine in its ability to maintain weight, or as a promoter of growth. Owing to variations in the amount of food assimilated the interpretation of feeding experiments must often be a matter of difficulty, and the criterion of the capacity of a substance to induce growth is its effect upon the animal as a whole. It may be assumed that the amino-acid requirements of the various organs and tissues of the body differ in accordance with differences in specific function, and with the chemical composition of their structural units, the cells of which they are composed. It has been suggested that histidine is necessary for nuclear synthesis [Rose and Cook, 1925], and the researches of Kossel [1928] indicate that arginine may be an important constituent of nuclear substance as in the case of sperm. Edlbacher and Merz [1927], have shown that the arginase content of tumours exceeds that of most normal tissues, and it may be noted that the arginine content of neoplastic tissue, as estimated by Sakaguchi's reaction [1915] is also relatively high. Therefore it appears that arginine is present in considerable amount in tumours, and that such tissues are well equipped for its catabolism, but unless they are capable of synthesising this amino-acid they must obtain it from their host; if arginine were present in excess of the nutritive requirements of the latter it would be available for the purpose of nuclear synthesis and cell proliferation, and this should be manifested by an increase in the rate of tumour growth.

It was the object of this investigation to determine whether arginine had any influence upon tumour growth-rate, and whether it was distinct from other amino-acids in exhibiting this effect.

MATERIAL.

The mice used for the experiments were ordinary, healthy, unselected stock, representing a mixed population; they were 2–3 months old at the time of implantation, and an approximately equal number of males and females were included in each experimental group. They were all fed alike on a diet of brown bread, flaked maize, and lettuce (when available). It may be of interest to note in connection with diet that the arginine content of brown bread, as estimated by Sakaguchi's reaction, is quite considerable, and both treated and control groups were given as much as they would eat.

The tumour was a transplantable mouse carcinoma, for which I am indebted to Mr J. J. M. Shaw, of the Department of Surgery, Edinburgh University. This tumour (M 63), is palpable about ten days after implantation, often earlier, and attains a large size in a period of three weeks. It produces no metastases, and therefore the mice remain perfectly well.

METHOD.

Implantation was performed by means of an implantation needle, strict aseptic precautions being observed.

The amino-acids[1] were administered by hypodermic injection at some distance from the site of implantation.

Exp. I. Implantation was carried out as described, and after ten days all mice bearing palpable tumours on that date were segregated, divided into groups, and treatment was commenced. This consisted in the injection of 0·3 cc. of a 10 % solution of the amino-acid allotted to the group, and treatment was repeated every 2 days until the termination of the experiment 22 days after implantation. On each day of treatment caliper measurements were taken of the tumours, and transferred direct to mm. squared paper; in this way it was an easy matter to measure the cross-sectional area of tumours of irregular shape. At the end of the experiment, size, *i.e.* cross-sectional area, was estimated, and from the tabulated results the mean was calculated for each group showing the increase during the experimental period. The ratio of the mean values on the 10th and 22nd days respectively was taken as an index of the rate of growth.

Exp. II. In this experiment the tumours used for implantation were known to be of lowered vitality (they were derived from tumours which had recurred after removal, and when implanted into fresh hosts had been found to exhibit a slow rate of growth, and a reduction in the usual percentage of "takes").

Two amino-acids were tested in this experiment for their ability to restore the vitality of this debilitated tissue. Treatment was begun 2 days before implantation, and continued every second day as in Exp. I, and caliper measurements were taken at the same intervals as before.

Special precautions were taken to ensure the uniformity of the implanted tissue in its distribution among the various groups, the following procedure being adopted; the mice were divided into three main groups, two treated, and one control, and each of these was subdivided into four small groups, *A*, *B*, *C*, *D*. One tumour was used to implant all the "*A*" groups, another for *B*, etc. In addition to this the mice were implanted in strict rotation as follows: mouse 1, Group I *A*; mouse 1, Group II *A*; mouse 1, Group III *A*; then mouse 2 in each group as in the first round.

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[1] Supplied by Messrs British Drug Houses, Ltd.

As an extra check upon the usual measurements all the tumours were weighed at the close of the experiment, the mean weight was calculated, and the ratio of each treated group to the control was compared with the ratio obtained by measurement of cross-section. The results will be found in Table III, Exp. II.

The results of these two experiments made it appear almost certain that arginine had a definite effect upon the rate of tumour growth; the variation in the growth potentiality of apparently healthy tumours observed in earlier work, however, had made it desirable to limit the number of animals used in each experiment, in order that the implanted tissue should be derived, as far as possible, from the same source. Consequently it seemed advisable to apply to the results the method described by Fisher [1928] for the determination of the significance of the difference of the means of small samples; the tabulated results therefore include the "*t*" and "*P*" values used in this method. According to Fisher [1928] we may "draw a conventional line at 0·05," *i.e.* if the value is at, or below, this figure the difference may be held to be significant, and would not have occurred by chance, and one may place "a high degree of confidence in the results" if $P = 0{\cdot}01$.

The choice of amino-acids was limited in accordance with the degree of their solubility in water. The low solubility of tyrosine prohibited its use for hypodermic injection; a considerable mortality attended the use of cystine for the same reason, and cysteine was substituted as a result. It was obviously impossible to test the guanidine radical of arginine separately [Barger, 1914], but sodium valerate was included in the series. Glutamic acid was given a separate trial in view of it being a constituent of glutathione; lysine was not tested.

An example of experimental data from which the results were calculated.

(Treatment begun 10 days after implantation of M 63.) Surface area of tumours as estimated by caliper measurements in mm.^{2}

Arginine:	Days since implantation						Ratio of size, day 10 : 22
	10	12	14	16	18	22	
1	16	64	106	154	250	527	1 : 33·0
2	60	118	193	290	332	614	1 : 10·2
3	64	126	166	274	443	714	1 : 11·1
4	96	119	205	254	254	419	1 : 4·3
5	96	156	198	212	260	303	1 : 33·1
6	10	42	58	173	237	430	1 : 43·0
7	23	57	105	175	207	242	1 : 10·5
8	8	24	75	175	260	353	1 : 44·7
9	7	28	36	96	143	216	1 : 30·8
10	4	6	74	131	176	246	1 : 61·5
11	6	6	131	180	210	437	1 : 72·9
12	6	8	137	183	225	257	1 : 32·1
Mean	33·0	62·8	123·6	174·7	249·0	396·0	1 : 29·7

After estimation of the probable error the mean values were respectively 33·0 ±6·6, and 396·0 ±29·5.

Controls:	Days since implantation						Ratio of size, day 10 : 22
	10	12	14	16	18	22	
1	11	17	32	32	57	80	1 : 7·3
2	44	66	102	137	196	288	1 : 6·5
3	56	79	121	180	197	257	1 : 4·9
4	5	15	19	39	40	49	1 : 9·9
5	33	67	79	102	159	179	1 : 5·4
6	50	66	99	217	372	497	1 : 9·9
7	13	48	57	82	114	184	1 : 14·1
8	13	16	31	58	74	157	1 : 12·2
9	7	21	21	33	84	265	1 : 37·8
10	47	82	113	113	119	277	1 : 6·0
11	14	24	31	40	51	84	1 : 6·0
12	60	143	171	291	291	336	1 : 5·6
Mean	29·0	46·2	69·8	100·3	146·1	221·0	1 : 10·4

After estimation of the probable error the mean values were respectively 29·0 ± 4·4 and 221·0 ± 24·4.

Difference of mean day 10 = 4·0 ± 0·3.
Difference of mean day 22 = 175·0 ± 3·2.

The value of t on day 10 = 0·3.
The value of P on day 10 = 0·9.

The value of t on day 22 = 3·2.
The value of P on day 22 = 0·01.

Experiment I.

Table I.

Group	No. in group	Size on 10th day: Difference of mean	t	P	Size on 22nd day: Difference of mean	t	P
Arginine Untreated	12 12	+ 4·0 ± 0·3	0·30	0·90	+ 175 ± 3·2	3·2	0·01
Glycine Untreated	10 7	± 0·0	0·30	0·70	− 28 ± 0·6	0·7	0·50
Alanine Untreated	12 7	+ 12·0 ± 1·3	1·00	0·30	+ 5 ± 0·09	0·1	1·00
Cystine Untreated	6 7	± 0·0	1·00	0·30	− 25 ± 0·3	1·4	0·20
Glutamic acid Untreated	10 7	+ 2·0 ± 0·7	0·60	1·00	− 14 ± 4·9	0·2	1·00
Arginine Untreated	4 7	+ 5·0 ± 0·7	0·14	0·90	+ 192 ± 4·2	3·4	0·01
Sodium valerate Untreated	11 6	− 8·0 ± 4·1	0·40	0·70	+ 54 ± 0·5	0·6	0·50
Arginine Untreated	4 6	+ 23·0 ± 0·7	0·90	0·40	+ 122 ± 2·1	1·6	0·10

The comparison of arginine and cystine is of value in view of the importance which has been attached to glutathione in connection with tumour growth. From the results obtained it would appear that neither cystine nor glutamic acid, a constituent of glutathione, has any appreciable effect upon the rate of tumour growth.

The results of the second experiment showed that histidine could not replace arginine as a stimulant of growth. It appeared to exert a certain effect upon the percentage of successful implantations, when tested on devitalised tissue, the number of failures being greater in the case of arginine than histidine, but

Table II. *Ratio of surface area of tumours ten and twenty-two days after implantation.*

Group	Ratio. Group mean	Difference of mean	*P*
Arginine	29·7 ± 14·3	+ 19·5 ± 0·84	0·01
Untreated	10·4 ± 6·0		
Glycine	17·9 ± 6·8	− 2·3 ± 0·17	0·70
Untreated	20·2 ± 6·3		
Alanine	15·1 ± 7·1	− 5·1 ± 0·36	0·40
Untreated	20·2 ± 6·3		
Cystine	21·4 ± 4·4	+ 1·2 ± 0·63	0·90
Untreated			
Glutamic acid	19·4 ± 7·5	+ 0·8 ± 0·05	1·00
Untreated	20·2 ± 6·3		
Sodium valerate	9·8 ± 2·2	+ 3·0 ± 0·11	0·15
Untreated	11·8 ± 3·5		
Arginine	15·0 ± 7·8	+ 8·2 ± 0·07	0·15
Untreated	11·8 ± 3·5		
Arginine	52·9 ± 5·7	+ 32·0 ± 2·60	0·01
Untreated	20·2 ± 6·3		

Table III. *Comparison of the effect of arginine and cystine, or cysteine, the latter being substituted for untreated controls.*

		Size on 10th day			Size on 22nd day		
Group	No. in group	Difference of mean	*t*	*P*	Difference of mean	*t*	*P*
Arginine	4	± 0·0	0·2	0·80	+ 217 ± 3·1	2·50	0·04
Cystine	6						
Arginine	8	+ 1·0 ± 0·16	0·1	0·80	+ 49 ± 0·8	0·90	0·35
Cysteine							

less in arginine-treated mice than in the untreated controls. The exact percentages of failures were, arginine 37·5 %, histidine 26 %, untreated 50 %. These figures are very high for implantation failures in the tumour M 63.

Experiment II.

Table I.

		Size on 10th day			Size on 22nd day		
Group	No. in group	Difference of mean	*t*	*P*	Difference of mean		*P*
Arginine	15	+ 14·0 ± 0·4	0·1	0·40	+ 134·0 ± 4·3	0·5	0·05
Untreated	12						
Histidine	17	+ 18·0 ± 0·1	0·1	0·10	+ 38·0 ± 0·8	1·7	0·10
Untreated	12						

Table II.

Group	No. in group	Ratio. Group mean	Difference of mean	*P*
Arginine	15	10·5 ± 7·47	5·5 ± 0·06	0·10
Untreated	12			
Histidine	17	6·8 ± 4·7	1·8 ± 0·06	0·40
Untreated	12			

Table III.

Group	No. in group	Mean wt.	Difference of mean	t	P
Arginine	15	1·6 ±0·27	0·9 ±0·21	1·5	0·1
Untreated	12	0·7 ±0·22			
Histidine	17	0·7 ±0·14	0·0	0·1	0·9
Untreated	12	0·7 ±0·22			

DISCUSSION.

From the tabulated results of Experiments I and II it can be seen that among the amino-acids tested for their effect upon tumour growth arginine alone induces a significant difference from the mean of the untreated control group on the 22nd day, as estimated by the value of P.

It is evident that if the size of tumours in a treated group exceeds that of the corresponding untreated group on the 10th day the former would be expected to exceed the latter on the 22nd day as well, apart from any treatment which the animals had received. In certain cases it may therefore appear that the difference of mean in the arginine-treated groups on the 22nd day was not so significant as it otherwise would seem. In order to assess the real value of the difference two points must be taken into consideration, of which one is the value of P. On the 10th day the P value indicates in every case that the difference in mean could be accounted for as the result of random sampling from a homogeneous population. The P value for the 22nd day, on the other hand, in three cases out of four of the arginine-treated groups is 0·05 or less, while the value for the groups treated with other amino-acids is in no case less than 0·1. The second point to be considered is the ratio of the sizes (cross-sectional area), on the 10th and 22nd days respectively within one and the same group. The mean ratio obtained after calculating the individual ratios in such a group can then be compared with its untreated control. The ratio will obviously be greater or less according to the mean size on the 10th day of the experiment unless the mean on the 22nd day is proportionately very great; since in some of the arginine-treated groups the mean was higher on the 10th day than it was in the controls the ratio might have been thus reduced, but in spite of this fact a markedly higher ratio occurs in the arginine-treated groups alone. The second experiment indicates that arginine and histidine have some slight effect in raising the percentage of "takes" in the case of debilitated tissue (as previously defined), but that only arginine has any appreciable influence upon its subsequent rate of growth. The difference in weight in these groups is not significant if judged by the value of P, but the mean weight is about 50 % greater in the arginine-treated group than in the untreated control, which agrees well with the difference in ratio where cross-sectional area was the criterion of size.

From this analysis of the data it seems justifiable to conclude that the influence of arginine upon tumour growth is real, and that it is specific in so far as a similar effect was not observed in the case of any other substance used in this series of experiments. It is suggested that an increase in nuclear substance leading to an acceleration of the rate of cell division, may be the mechanism through which the observed increase in size is attained. Certain inferences concerning the biology of tumour growth suggest themselves, assuming this hypothesis to be correct, but the consideration of theoretical implications is beyond the scope of the present paper, and the facts are therefore recorded alone.

SUMMARY.

A comparison of the effects upon tumour growth of certain amino- and fatty-acids (arginine, histidine, glycine, alanine, cystine, cysteine, glutamic acid, and sodium valerate), showed that arginine was the only substance investigated which exerted a definite effect upon the rate of tumour growth.

In an experiment dealing with the implantation of "devitalised" tissue it was found that histidine appeared to increase the percentage of "takes." It had no effect upon the subsequent rate of growth, whereas arginine had a definitely stimulating effect.

I wish to express my thanks to Prof. Crew for the facilities provided for the prosecution of this research, and for much helpful criticism and advice, and I would also acknowledge my indebtedness to Dr Kermack, of the Royal College of Physicians, for his advice in the statistical treatment of the results.

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LXVII. A STUDY OF THE ACID-BASE EQUILIBRIUM OF THE OCULAR HUMOURS.

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So long as the fluids in the eye were regarded as the secretory product of the ciliary body no direct relationship between them and the blood stream was postulated. A large amount of experimental work, however, has recently been brought forward which tends to show that this fluid is a dialysate of the capillary plasma [see Duke-Elder, 1927, 1], and if this theory is correct the relationship between the intra-ocular fluid and the blood-plasma should be very intimate. Most of the evidence which has been brought forward in support of the newer theory is either chemical in nature, dealing with the constitution of normal and abnormal aqueous humour, or physical, dealing with the hydrostatic, osmotic and electrostatic forces involved. It may be of value therefore to consider how far the relationship of the acid-base equilibrium of the fluids in the eye with that of the plasma brings support to the more recent conceptions.

It was considered desirable to estimate the hydrogen ion concentration of the vitreous and aqueous humours, the carbon dioxide content of the aqueous humour, and the carbon dioxide content and capacity as well as the dissolved oxygen in the vitreous humour. The eyes were obtained immediately after death and the aqueous humour was abstracted by means of a syringe and kept under paraffin oil. The vitreous humour was then shelled out through a posterior incision and was kept in a paraffin-covered tube.

Since the vitreous humour is difficult to measure accurately on account of its jelly-like consistency, it was used after liquefaction by forcing it through the perforated plunger of a syringe the opening of which had been plugged.

p_H DETERMINATION.

Cullen's [1922] colorimetric method as applied in Myers's [1924] technique was employed. Since both humours are only slightly buffered in comparison with the blood-plasma, the p_H determinations were carried out without any dilution. The values are only comparative in respect to the two humours, since no corrections were made for temperature (Tables I and II).

Table I. *Aqueous humour.*

p_H *values of the aqueous humours of cattle varying in age from 5 weeks to 2 years.*

No. of animal	Age of animal	Room temp.	p_H		
			Eye 1	Eye 2	Average
1	5 wks.	26°	7·20	7·25	7·23
2	2 mths.	27	Mixture		7·22
3	4 ,,	30	7·43	7·33	7·38
4	4 ,,	27·5	7·40	—	7·40
5	4 ,,	27	7·27	7·32	7·30
6	4 ,,	27	Mixture		7·41
7	8 ,,	27	7·33	7·44	7·39
8	1½ yrs.	24	7·42	7·44	7·43
9	1½ ,,	24	7·40	7·41	7·41
10	1½ ,,	24	7·38	—	7·38
11	1½ ,,	25·5	*7·35	7·35	7·35
12	1½ ,,	25·5	*7·10	7·20	7·15
13	1½ ,,	25·5	*7·35	7·25	7·30
14	1½ ,,	25·5	*7·42	7·36	7·39
15	1½ ,,	26	*7·25	7·20	7·23
16	1½ ,,	26	*7·36	7·20	7·28
17	1½ ,,	26	*7·35	7·33	7·34
18	2 ,,	27·5	7·28	7·26	7·27
19	2 ,,	27·5	7·35	7·34	7·35
20	2 ,,	26	7·31	7·40	7·36
21	2 ,,	26	7·20	—	7·20
22	2 ,,	26	Mixture		7·40
23	2 ,,	26	7·58	7·48	7·53
24	2 ,,	26	7·48	7·58	7·53
25	2 ,,	25·5	7·39	7·44	7·32
26	2 ,,	25·5	7·51	7·42	7·47
27	2 ,,	25·5	7·45	7·45	7·45
28	2 ,,	25	*7·28	7·33	7·31
29	2 ,,	25	*7·20	7·25	7·23
30	2 ,,	25	*7·27	7·25	7·26
31	2 ,,	25	*7·18	—	7·18
32 (Sheep)	Age unknown	27	7·23	—	7·23

* The humours were allowed to remain in the extracted eye in the refrigerator half a day. All other results were obtained from strictly fresh humours.

When the ocular humours are allowed to remain in the excised eyes for some hours, they become more acid (see Tables III and IV), despite a possible loss of carbon dioxide, which may be due to autolytic decomposition of protein. Unpublished earlier analyses by the author (1927), in confirmation of the findings of other workers, revealed that the vitreous humour under these conditions suffers a great increase in protein content. This increase in protein content is thought to be due to transudation from the surrounding capillaries [Duke-Elder, 1927, 2].

A comparison of the average p_H values of aqueous humours of cattle for different age groups with those of vitreous humours for similar age groups shows that the aqueous humour is slightly less alkaline than the vitreous humour. This is illustrated in Fig. 1.

Table II. *Vitreous humour.*

p_H *values of the vitreous humours of cattle varying in age from* 3 *months to* 2 *years.*

				p_H			
				Determinations			
No. of animal	Age of animal	Age of specimen under oil (days)	Room temp.	1	2	3	Average
1	3 mths.	Fresh	27°	7·27	7·20	7·31	7·26
2	4 ,,	,,	27·5	7·40	7·41	—	7·40
3	8 ,,	,,	27	7·36	7·43	—	7·40
4 Eye 1	1½ yrs.	15	24	*7·36	7·33	—	7·35
Eye 2	1½ ,,	15	24	*7·33	7·31	—	7·32
5	1½ ,,	15	24	*7·30	7·30	7·15	7·30
6	1½ ,,	16	22	*7·14	7·20	7·15	7·16
7	1½ ,,	4	22·5	*7·24	7·24	—	7·24
8 Eye 1	1½ ,,	4	22·5	*7·24	7·24	—	7·24
Eye 2	1½ ,,	4	22·5	*7·25	7·30	—	7·27
9	1½ ,,	3	25	7·45	—	—	7·45
10	1½ ,,	3	25	7·54	—	—	7·54
11	1½ ,,	3	25	7·49	—	—	7·49
12	1½ ,,	3	25	7·44	—	—	7·44
13	1½ ,,	3	25	7·40	7·40	—	7·40
14	1½ ,,	3	25	7·40	—	—	7·40
15	1½ ,,	3	25	7·35	7·36	—	7·36
16 Eye 1	2 ,,	Fresh	25	7·44	7·38	—	7·41
Eye 2	2 ,,	,,	25	7·38	7·42	—	7·40
17 Eye 1	2 ,,	1	24	7·44	7·40	7·45	7·43
Eye 2	2 ,,	1	24	7 40	7·40	7·38	7·39
18 Eye 1	2 ,,	2	26	7·49	7·54	7·54	7·52
Eye 2		2	26	7·49	7·56	7·54	7·53
19	2 ,,	2	26	7·66	7·65	7·65	7·65
20 Eye 1	2 ,,	2	26	7·64	7·58	7·64	7·61
Eye 2		2	26	7·63	7·61	—	7·62
21 Eye 1	2 ,,	6	25·5	*7·49	7·54	—	7·51
Eye 2		6	25·5	*7·24	7·33	7·38	7·32
22 Eye 1	2 ,,	6	26	*7·40	7·43	—	7·41
Eye 2		6	26	*7·32	7·33	—	7·33
23	2 ,,	6	26	*7·46	7·36	—	7·41
		6	26	*7·35	7·40	7·35	7·37

* The humours were allowed to remain in the excised eye for half a day in the refrigerator.

Table III. p_H *values for fresh aqueous humours and for those kept in the excised eye, in the refrigerator, for half a day. (The specimens were handled under oil.)*

Fresh aqueous		Aqueous kept in the extracted eye for half a day	
No. of animal	p_H averages	No. of animal	p_H averages
	Animals 6 months old.		
8	7·43	11	7·35
9	7·41	12	7·15
10	7·38	13	7·30
—	—	14	7·39
—	—	15	7·23
—	—	16	7·28
—	-	17	7·34
Average	7·41		7·29
	Animals 2 years old.		
18	7·27	29	7·28
19	7·35	30	7·20
20	7·36	31	7·27
21	7·20	32	7·18
22	7·40	—	—
23	7·53	—	—
24	7·53	—	—
25	7·42	—	—
26	7·47	—	—
27	7·45	—	—
Average	7·40		[illegible]

Table IV. p_H *values for fresh vitreous humours and those kept in the excised eyes, in the refrigerator, for half a day.* (*All were handled under oil.*)

Vitreous removed immediately		Vitreous kept in the extracted eye for half a day	
No. of animal	p_H averages	No. of animal	p_H averages
	Animals 1½ years old.		
9	7·45	4	7·35
10	7·54	4	7·32
11	7·49	5	7·30
12	7·44	6	7·16
13	7·40	7	7·24
14	7·40	8	7·24
15	7·36	8	7·24
Average	7·44		7·27
	Animals 2 years old.		
16	7·41	21	7·51
16	7·40	21	7·32
17	7·43	22	7·41
17	7·39	22	7·33
18	7·52	23	7·41
18	7·53	23	7·37
19	7·65		
20	7·61		
20	7·62		
Average	7·51		7·39

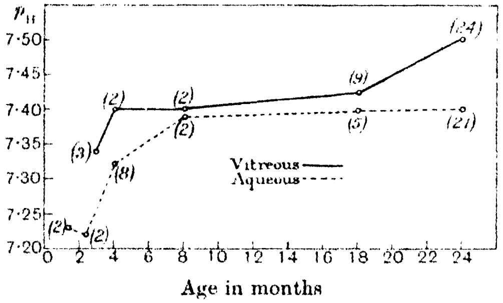

Fig. 1. Rate of increase in alkalinity of the ocular humours of cattle with advancing age. (Figures in parentheses denote the number of eyes on which the averages were based.)

CARBON DIOXIDE DETERMINATIONS.

Analyses for carbon dioxide were made by the method of Van Slyke and Neill [1924], in which 2 cc. of the fluid were used.

The vitreous humour only was studied in respect to the relationship of the carbon dioxide content to the age of the animal. The carbon dioxide content of the vitreous humour decreases with increasing age. This is shown by the arrangement of the data in Table V and particularly by the average values for various ages presented in Table VI.

The aqueous humour was analysed for its carbon dioxide content only (see Table VIII). In the vitreous humour both carbon dioxide content and capacity were studied. The carbon dioxide capacity determination differed from the

Table V. *Carbon dioxide content of the vitreous humours from cattle varying in age from 6 weeks to 2 years.*

No. of animal	Age of animal	No. of eye	Age of specimen under oil (days)	Vol. % CO_2 content			
				Determinations			
				1	2	3	Average
1	6 wks.	1	Fresh	58·7	58·5	59·5	58·9
		2	,,	58·1	—	—	58·1
2	2 mths.	Mixture	2	74·1	74·3	—	74·2
3	3 ,,	,,	Fresh	60·8	60·1	—	60·5
4	4 ,,	,,	,,	52·8	52·1	52·0	52·3
5	8 ,,	,,	,,	59·5	59·9	—	59·7
6	1 yr.	,,	,,	54·9	—	—	54·9
7	1 ,,	,,	,,	54·9	—	—	54·9
8	1 ,,	,,	,,	62·3	—	—	62·3
			3	60·9	59·9	—	60·4
			7	60·8	—	—	60·8
9	1½ yrs.	,,	4	46·2	46·5	—	46·4
10	1½ ,,	,,	,,	44·1	46·2	44·7	45·0
11	1½ ,,	,,	,,	41·2	42·2	—	41·7
12	2 yrs.	1	Fresh	51·3	51·5	50·7	51·1
		2	3	52·4	—	—	52·4
13	2 ,,	1	1	45·4	44·3	44·5	44·7
		2	,,	45·1	45·1	—	45·1
14	2 ,,	1	Fresh	46·3	46·7	—	46·5
15	2 ,,	1	,,	46·3	46·0	—	46·2
		2	,,	44·7	—	—	44·7
16	2 ,,	1	,,	45·6	46·4	—	46·0
17	2 ,,	1	,,	46·3	—	—	46·3
		2	,,	47·1	46·3	—	46·7
18	2 ,,	1	15	38·0	37·3	—	37·7
		2	15	37·3	37·3	—	37·3
19	2 ,,	1	15	38·3	—	—	38·3
20	2 ,,	1	16	40·8	40·6	—	40·7
21	2 ,,	1	2	48·5	—	—	48·5
		2	2	54·5	—	—	54·5
22	2 ,,	1	2	55·4	—	—	55·4
23	2 ,,	1	2	43·5	—	—	43·5
24	2 ,,	1	6	42·7	40·8	—	41·8
25	2 ,,	1	6	44·8	—	—	44·8
26	2 ,,	1	6	43·7	—	—	43·7
27	2 ,,	1	6	44·7	—	—	44·7
28	2 ,,	1	6	47·1	—	—	47·1
29	2 ,,	1	Fresh	47·5	47·4	—	47·5
		2	,,	45·7	47·1	—	47·4
30	2 ,,	1	1	43·8	42·3	—	43·1
		2	1	45·2	45·6	—	45·4

Table VI. *Average values of the carbon dioxide content of vitreous humours of different ages.*

Age	6 wks.	2 mths.	3 mths.	4 mths.	8 mths.	1 yr.	1½ yrs.	2 yrs.
Vol. % of CO_2	58·7	74·2	60·5	52·3	59·7	59·0	44·4	42·9
No. of animals used	1	1	1	1	1	3	3	19

carbon dioxide content determination in that in the former analysis the vitreous humour was equilibrated with alveolar air before the carbon dioxide was withdrawn. Under these conditions the vitreous humour took up no additional carbon dioxide. Therefore the carbon dioxide content and capacity of the vitreous humour are alike. The results are given in Table VII.

Table VII. *The carbon dioxide content compared with the carbon dioxide capacity of the vitreous humour.*

			Vol. % CO_2 content				Vol. % CO_2 capacity		
			Determinations				Determinations		
No. of animal	Age of animal	Age of specimen	1	2	3	Average	1	2	Average
4	4 mths.	Fresh	52·8	52·1	52·3	52·3	51·7	52·3	52·0
12	2 yrs.	3 days	52·4	—	—	52·4	51·4	51·9	51·7

Table VIII. *Aqueous humour.*

The carbon dioxide content of fresh aqueous humour compared with that previously used in a colorimetric p_H *determination. (The humours in both cases were obtained from cattle 1½–2 years old, and handled under oil.)*

	Vol. % CO_2 content				
		After p_H determination			
		Determinations			
No. of animal	Original sample	1	2	3	Average
1	49·2	—	—	—	—
2	49·4	47·8	—	—	47·8
3	47·6	47·5	—	—	47·5
4	48·9	—	—	—	—
5–8	40·3	36·8	—	—	36·8
9–11	—	37·9	38·7	38·0	38·2
12–13	45·1	39·5	39·7	—	39·6
14–16	47·9	—	—	—	—
14	—	38·9	—	—	38·9
15	—	50·4	—	—	50·4
16	—	40·2	—	—	40·2
17	—	42·4	—	—	42·4
17–18	—	40·8	—	—	40·8
19	53·5	—	—	—	—
20	45·7	—	—	—	—
Average	47·5				42·3

Table IX. *Vitreous humour.*

The carbon dioxide content of fresh vitreous humour compared with that of vitreous humour previously used in a colorimetric p_H *determination. (The humours in both cases were obtained from cattle 1½–2 years old, and handled under oil.)*

		Vol. % CO_2 content; averages	
No. of animal	Age of specimen (days)	Original	After p_H det.
21	2	48·5	46·3
21	2	54·5	56·0
22	2	55·4	53·4
23	2	43·5	42·4
24	6	41·8	38·2
25	6	44·8	44·5
26	6	43·7	41·5
27	6	44·6	43·5
27	6	47·1	42·3
	Average	47·1	45·3

Carbon dioxide content was determined on several samples of both humours after p_H determinations had been carried out on them, and the results were compared with those obtained on the same humours in their original fresh state. The results given in Tables VIII and IX show that both aqueous and vitreous humours tend to lose their carbon dioxide, if disturbed, even under oil, the aqueous humour more so than the vitreous humour.

Several determinations for carbon dioxide content of the plasma and the vitreous humour in the same animal show that the carbon dioxide content of the vitreous humour is lower than that of the plasma.

Table X. *The carbon dioxide content of ox-plasma as compared with the carbon dioxide content (or capacity, see Table VII) of the vitreous humour of the same animals. The figures express average values.*

No. of animal	Age of animal (yrs.)	Vol. % CO_2 plasma	Vol. % CO_2 vitreous
12 and 13	2	59·1 (2)*	47·8 (9)*

* This figure indicates the number of determinations on which the average is based.

Comparing the figure 47·5 vol. % obtained on fresh aqueous humour of cattle $1\frac{1}{2}$–2 years old (Table VIII) with 43·7 vol. %, the average figure for carbon dioxide content of fresh vitreous humour of cattle of the same age (Table V), it is seen that the carbon dioxide content of the aqueous exceeds that of the vitreous humour by 3·8 vol. %. Hence the order of increasing values of carbon dioxide content is represented in the ocular humours and the blood-plasma of cattle of the same age, as follows: vitreous, 43·7 vol. %; aqueous 47·5 vol. %; plasma 59·1 vol. %.

Table XI. *The carbon dioxide content of fresh vitreous humour as compared with vitreous preserved in the refrigerator under and without oil.*

		Vol. % CO_2 content; averages		
No. of animal	Age of animal (yrs.)	Fresh	Preserved under oil	Days in refrigerator
8	1	62·3	60·5	3–7
12	2	51·1	52·4	3
			Preserved without oil	
14	2	46·5	46·7	3–7

The carbon dioxide content of the vitreous humour was studied also after the humour had been kept in the refrigerator for several days, both without oil and under oil. The figures presented in Table V, and the results shown in Table XI, indicate that the vitreous humour, whether under oil or exposed, can be kept in the refrigerator for about a week without loss of carbon dioxide. The average of the nine determinations made on the fresh vitreous humours from 2-year old animals in Table V is 46·9 vol. %. The average of the fourteen determinations on similar vitreous humours that had been kept in the refrigerator from one to six days is 46·1 vol. % (Table V). A detectable loss of carbon dioxide occurs, however, when the humour, even under oil, is allowed to stand for longer than a week, as shown by the results on animals 18, 19 and 20 in Table V.

Oxygen determinations on the vitreous humour.

The analyses were made by the Van Slyke and Neill [1924] method, in which 2 cc. of the fluid were employed.

It was thought probable that the vitreous humour would contain little, if any, oxygen, except the traces in physical solution. Table XII proves this conjecture to be correct.

Table XII. *Analysis of the vitreous humour for dissolved oxygen.*

No. of animal	No. of determinations	Mm. Hg before absorption of O_2	Mm. Hg after addition of $Na_2S_2O_4$	Difference in mm. Hg
1	1	116·5	116·2	+0·3
1	2	114·0	116·5	-2·5
1	3	109·0	110·8	-1·8
1	4	108·5	111·0	-2·5
2	5	115·0	118·0	-3·0

A blank determination gave a figure of 0·5 mm. Hg. When this figure is subtracted from those of the last column, a negative value is obtained for oxygen in each case. Any amount of oxygen larger than the equivalent of experimental error would be represented under the assumed conditions by a positive value. The fact that all the five values happen to be negative suggests that some other gas was evolved on addition of $Na_2S_2O_4$ and proves (assuming the amount of this gas to be negligible) that the vitreous humour contains no dissolved oxygen. The gas evolved was identified, by its odour, as sulphur dioxide.

DISCUSSION.

The carbon dioxide content in order of decreasing values is represented by the fluids of cattle 1½–2 years old as follows: plasma, 59·1 vol. %; aqueous humour, 47·5 vol. %; vitreous humour, 43·7 vol. %.

No direct experimental data are available which would show whether changes in the hydrogen ion concentration of the blood are reflected in the ocular humours as shown in respect to sugar and carbon dioxide. The present work brings out the fact that the vitreous humour is slightly more alkaline than the aqueous humour. This is to be explained from the fact that the vitreous humour contains at least twice as much protein as the aqueous humour. Duke-Elder [1927, 3] has shown that the aqueous humour is particularly rich in the acid radicals (Cl^-, $PO_4^{\equiv}$, $SO_4^{=}$) of the inorganic salts present in the body fluids, whereas the blood-plasma exceeds the aqueous humour in respect to the cations (Na^+, K^+, Ca^{++}, Mg^{++}) of the same salts. It is an accepted fact that the cations are held more or less in combination by the blood-proteins, and thus partially withdrawn from body fluids that contain less protein toward those that contain more. It is to be expected therefore that the plasma, since it contains more protein than the vitreous humour, must be more alkaline than the latter. According to data obtained by Baurmann [1927], the p_H values of human aqueous humours vary between 7·20 and 7·35, with an average of 7·27. These figures are definitely lower than those for blood, which are 7·35 to 7·50. The data of the present work show, on the whole, smaller differences between the aqueous and vitreous humours of cattle than Baurmann observed between plasma and aqueous humour in man. It is clear that the order of increasing values of the hydrogen ion concentration (or decreasing alkalinity) on the p_H scale is represented by the ocular humours and the plasma as follows: p_H of plasma $>$ p_H of vitreous humour $>$ p_H of aqueous humour.

In view of the foregoing, there is sufficient reason to postulate that changes in the hydrogen ion concentration of the blood would be readily reflected in the ocular humours, particularly in the aqueous humour.

SUMMARY.

(1) Carbon dioxide content and capacity are identical for the vitreous humour. The amount decreases, with a few fluctuations, on the average from 58·7 vol. % in a calf 6 weeks old to 42·9 vol. % in cattle 2 years old. Plasma exceeds the vitreous humour in carbon dioxide capacity by an average of 11 vol. % in 2-year old cattle. The vitreous humour, in its natural state, can be kept under oil in the refrigerator for about 1 week without loss of carbon dioxide. If, however, its structure is destroyed by mechanical liquefaction, it tends to lose its carbon dioxide much more readily.

(2) No dissolved oxygen could be demonstrated in the vitreous humour.

(3) The aqueous humour of cattle 1½–2 years old contained an average of 47·5 vol. % carbon dioxide. On the other hand, an average figure of 42·3 vol. % was obtained on the same or similar humours after they had been used in a colorimetric p_H determination. Hence, the aqueous humour loses its carbon dioxide very readily if disturbed, even under oil.

(4) The carbon dioxide content of the aqueous humour exceeds that of the vitreous by 3·8 vol. % for cattle 1½–2 years old, the average figures being 47·5 vol. % and 43·7 vol. % respectively.

(5) There is a distinct decrease in the hydrogen ion concentration of the aqueous humour of cattle with advancing age. The relative p_H values increase from 7·23 in a 5-week old calf to 7·40 in 2-year old cattle. (No correction was made for temperature.)

(6) The relative p_H values of the vitreous humour of cattle increase, on the average, from 7·29 in 3-month old calves to 7·50 in 2-year old cattle.

(7) The aqueous humour is slightly, but consistently, more acid than the vitreous humour.

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LXVIII. THE INACTIVATION OF TRYPSIN BY HEAT.[1]

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The study of the heat-inactivation of trypsin contained in a crude extract of dried pancreas gland powder is complicated by the "spontaneous activation" which occurs in such extracts. Waldschmidt-Leitz and Harteneck [1925] have recently interpreted this phenomenon as due to the production of enterokinase from its pre-stage, which occurs together with the trypsin in the pancreas. This transformation takes place in aqueous extracts of dried pancreas powder, or in the dried powder itself on prolonged storage. As a consequence, in an aqueous extract of dried pancreas powder maintained at constant temperature two processes occur, heat-inactivation of the trypsin and simultaneously "spontaneous activation" of the enzyme. Thus, if the trypsin content of the extract be measured from time to time by hydrolysis of a suitable protein, there may be, instead of the decrease to be expected from heat-inactivation of the enzyme, an apparent increase in trypsin content, if it so happens that the "spontaneous activation" effect preponderates over the heat inactivation. Furthermore, Waldschmidt-Leitz has indicated that trypsin associated with its activator is less stable than the enzyme alone. In view of this the main experiments described in this paper have been carried out with a purified extract of the dried pancreas powder, in which the trypsin is free from enterokinase and its pre-stage.

EXPERIMENTAL PROCEDURE.

To study the inactivation of trypsin at different p_H values the following procedure was adopted. To 10 cc. of the neutral solution prepared in the manner described below were added normal acetic acid or normal ammonia to bring it to a required p_H and then distilled water was added to make the total volume 11 cc. This volume of solution was divided out into a number of test-tubes already immersed in the thermostat, regulated to ± 0·05°, at 50°. The test-tubes were then tightly stoppered and removed at various time intervals from the thermostat into crushed ice. The trypsin content of 1 cc. of solution was then measured. For the major portion of the work the trypsin content was measured by the method of Willstätter *et al.* [1926] (activation with enterokinase and subsequent interaction with caseinogen at p_H 8·7 at 30° for 2 minutes). While the work was in progress a modification of this method was published by Linderstrøm-Lang and Steenberg [1929]. (This modification provides for a more definite control of the activation process with enterokinase in the analytical procedure. A buffer is used and the activation is always carried out with an arbitrarily chosen "enterokinase unit." Further, the hydrolysis is made to take place at p_H 9·1.) Accordingly the measurements, already made by the writer for the determination of the critical increment of the inactivation process, were repeated using this revised technique and substantially the same results were obtained.

Preparations.

(*a*) *Preparation of trypsin free from enterokinase and the pre-stage of the activator.* In the earlier work of the Willstätter school a separation of trypsin from the activator and its pre-stage was accomplished by a series of selective adsorptions on alumina $C_γ$. Later a revised method and technique was published by Waldschmidt-Leitz and Linderstrøm-Lang [1927]. The principle of the latter method is to adsorb trypsin-kinase and the pre-stage of the activator on caseinogen, and then, by precipitating the protein, to leave the trypsin behind in the mother liquor. This process of purification, due to Waldschmidt-Leitz and Linderstrom-Lang was carried out as follows.

Pig's pancreas, free from fat, was prepared by drying with acetone and ether according to the procedure of Willstätter and Waldschmidt-Leitz [1923]. 6 g. of the dried gland powder were added to 100 cc. of glycerol-water mixture (containing 80 % glycerol). The powder was dispersed through the liquid by shaking and the dispersion kept for 5 hours at 30°. The finely divided suspension was centrifuged, and the supernatant liquid finally filtered. To 25 cc. of the clear ice-cold glycerol extract prepared in this manner were added 15 cc. of an ice-cold 6 % caseinogen solution. The mixture was left for 5 minutes in the ice-chest and was then treated with 2 cc. of *N* acetic acid. The resulting precipitate of protein was filtered off and to 20 cc. of the filtrate was added a further 15 cc. of ice-cold 6 % caseinogen solution followed by 1 cc. of *N* acetic acid. The precipitate was again filtered off. The filtrate, now clear and colourless, was brought to neutrality by addition of *N* ammonia. This neutral solution, which contains the trypsin completely free[1] from enterokinase and its pre-stage, was kept in the ice-chest.

(*b*) *Enterokinase solution.* Pig's intestinal mucosa was treated with acetone, acetone-ether mixture, and finally ether according to the procedure of Waldschmidt-Leitz [1924]. An aqueous extract was prepared by suspending 6 g. of the dried mucosa in 200 cc. distilled water for 5 hours at 30°. The suspension was centrifuged and filtered. To 100 cc. of the clear filtrate were added 1·6 cc. N acetic acid and the resulting precipitate of protein matter was centrifuged off. The clear supernatant liquid was neutralised with N ammonia and kept in the ice-chest.

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[1] This statement is borne out by the fact that there was no hydrolysis of caseinogen in 2 hours at 30°.

(*c*) *Caseinogen solution.* The caseinogen solution was prepared by addition of 100 cc. 0·05 N ammonia to 6 g. caseinogen (Kahlbaum-Hammarsten), stirring and keeping for 1 hour at 30°. This solution is referred to as a 6 % caseinogen solution.

(*d*) *Buffer solutions.* For the method of determination according to Willstätter *et al.* [1926] the buffer solution was made up by mixing equal volumes of N ammonia and N ammonium chloride. The p_H of this buffer was measured at 20° with the glass electrode and found to be 8·6.

For the Linderstrøm-Lang and Steenberg modification of the method two buffer solutions were made up as follows:

(i) by mixing 300 cc. N ammonium chloride and 50 cc. N ammonia;

(ii) by mixing 250 cc. N ammonia and 350 cc. water.

The p_H measurements of the trypsin solution were made by means of the glass electrode at room temperature.

The course of the reaction.

The heat-inactivation of a number of enzymes has been studied by various investigators and in general it has been found that the course of the reaction is "unimolecular." Thus Tammann [1895] found that the heat-inactivation of emulsin conforms to the unimolecular expression, and Madsen and Walbum [1907] obtained the same result for the proteolytic enzymes pepsin and trypsin.

It should be emphasised that all previous work on the heat-inactivation of trypsin has been carried out with material in which the enzyme has been associated with the activator, enterokinase. Since trypsin associated with the activator, *i.e.* trypsin-kinase, and non-activated trypsin are quite specific in their function as catalysts, it cannot reasonably be assumed that the loss of their catalytic efficiency on heating will occur at the same rate. Consequently previous work on the enzyme is not strictly comparable with the results described in this paper obtained with purified trypsin solution. Furthermore, most of the previous work has been carried out with unpurified material containing a considerable amount of protein matter. In such enzyme material, which contains the trypsin associated with enterokinase and therefore endowed with the capacity to hydrolyse native proteins, it is to be expected that hydrolysis of the protein matter contained in the enzyme preparation will proceed during the course of the inactivation. Thus at least two factors are introduced which may influence the temperature sensitivity of the enzyme, namely:

(1) a change in p_H, due to the formation of free carboxyl and amino-groups;

(2) the formation of reaction products which have a stabilising influence upon the enzyme, as the work of Schmidt [1911], Vernon [1904] and Northrop [1922] has shown.

Although the results obtained by Tammann and by Madsen and Walbum were in agreement with the unimolecular equation this is not the experience of other workers. Thus Northrop [1922], working with "Fairchild's trypsin," found that the course of the heat-inactivation did not conform to the unimolecular expression. He suggested that the observed decrease in the unimolecular constants with time is due to the enzyme acting upon the protein matter in the preparation used and thus forming a compound with the products of reaction which is more stable to heat than the "free" enzyme. On purifying the enzyme solution by dialysis he obtained, in one experiment, a satisfactory unimolecular constant. Vernon [1904], working with a pancreas extract containing activator, had also previously found that the constants worked out on the unimolecular basis decreased with time. He ascribed this falling off as due to the presence of several enzymes of different degrees of stability. In the light of the more recent work it is difficult to accept this explanation, for Vernon measured the quantity of enzyme by the hydrolysis of fibrin, and therefore was actually measuring the quantity of one enzyme only, namely activated trypsin, since non-activated trypsin and erepsin do not possess the property of hydrolysing native proteins.

In the experiments described in this paper, with trypsin freed from enterokinase and its pre-stage, it is found that the course of the heat-inactivation in fact follows the unimolecular expression. It would appear, as Northrop suggested, that the decrease in the unimolecular constants observed with unpurified trypsin solutions is due to the protective influence of the substances formed by the action of trypsin-kinase upon the protein in the solution.

The applicability of the unimolecular expression for the heat-inactivation of the trypsin solution employed by the writer is illustrated in Table I, for solutions at p_H values in the alkaline, acid and neutrality regions respectively.

The measurements at a particular time interval were done in duplicate and in all the following tables the magnitude of the "trypsin unit per cc." set down opposite a particular time is the mean of such duplicate experiments.

The values of k_{uni} were obtained from the equation:

$$k_{uni} = \frac{2{\cdot}303}{t} \log_{10} \frac{a}{a-x}$$

where a = trypsin units per cc. at zero time,

$a - x$ = trypsin units per cc. after time t mins.

The trypsin unit employed is that defined by Willstätter *et al.* [1926], namely the quantity of trypsin which produces in a 6 % caseinogen solution under the standard conditions of measurement an increase in acidity equivalent to 1·05 cc. 0·2 N NaOH.

Table I.

Temp. 50°. Glycerol content of solution 24 %.

Time t mins	Trypsin units per cc.	k_{uni}
	p_H 8·40.	
0	1·60	—
60	1·25	$3{\cdot}97 \times 10^{-3}$
129	1·05	$3{\cdot}27 \times 10^{-3}$
161	0·905	$3{\cdot}54 \times 10^{-3}$
211	0·78	$3{\cdot}40 \times 10^{-3}$
	p_H 5·86.	
0	1·52	—
60	1·28	$2{\cdot}86 \times 10^{-3}$
124	1·07	$2{\cdot}82 \times 10^{-3}$
159	0·94	$3{\cdot}02 \times 10^{-3}$
	p_H 7·12.	
0	1·60	—
120	1·22	$2{\cdot}26 \times 10^{-3}$
240	0·93	$2{\cdot}24 \times 10^{-3}$
360	0·77	$2{\cdot}04 \times 10^{-3}$

THE EFFECT OF p_H UPON THE HEAT INACTIVATION.

The p_H of the medium has a very marked effect upon the stability of all enzymes. In general it is found that each enzyme has a fairly broad optimum region of stability and that outside this region the rate of destruction increases rapidly. In Table II the results obtained for the heat-inactivation of trypsin at various p_H values are summarised. The mean of the values for k_{uni} at a particular p_H are set down opposite that p_H value.

These results are plotted in Fig. 1, where it is seen that the enzyme is most stable at p_H 6·5.

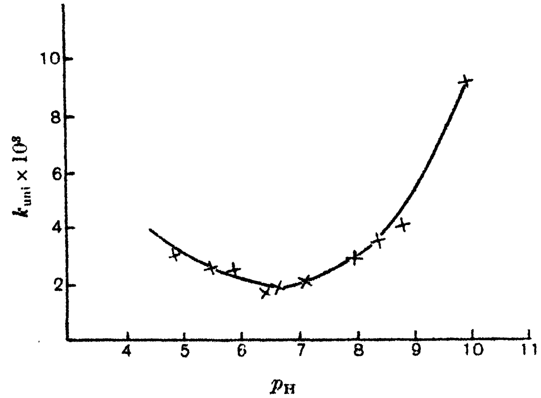

Fig. 1.

Table II.

Temp. 50°. Glycerol content of solution 24 %.

p_H	$k_{\text{uni}} \times 10^3$	p_H	$k_{\text{uni}} \times 10^3$
9·95	9·23	6·64	2·00
8·82	4·12	6·51	1·70
8·40	3·59	5·86	2·90
7·96	2·98	5·52	2·64
7·12	2·18	4·85	3·14

It is not possible to compare with certainty these results obtained for the effect of p_H with other work in which a different enzyme material has been employed. This point has been emphasised by Willstätter, Haurowitz and Memmen [1924] for the dependence of enzyme activity upon p_H, where they show that substances of various kinds may modify the influence of p_H. At the same time it is noteworthy that for many enzymes the region of optimum stability appears to lie about p_H 6. Thus Morgulis and Beber [1928] found that the optimum stability for catalase lies between p_H 6 and 6·5, Michaelis and Rothstein [1922] found the optimum stability for pepsin at p_H 6, whilst Ernstrom [1922] found an optimum stability at p_H 6·1 for salivary amylase, and Willstätter, Graser and Kuhn [1922] found the optimum stability of saccharase at p_H 5·8. Northrop [1922] found the optimum stability for the trypsin he used to be at p_H 5·0.

The shape of the p_H-stability curve is somewhat similar to that obtained by Lewis [1926] for the effect of p_H upon the rate of heat-denaturation of proteins.

THE CRITICAL INCREMENT OF THE PROCESS.

The critical increment for the process of heat-inactivation of enzymes is found to be very large and comparable with that found for the heat-denaturation of proteins by Chick and Martin [1910] and Lewis [1926].

Experiments were carried out to determine the critical increment for the heat-inactivation of trypsin. This was determined by measuring k_{uni} at 50° and 60° and then substituting the mean values in the equation:

$$\frac{d \ln k_{uni}}{dT} = \frac{E}{RT^2}.$$

Measurements were made in the three cases:

(1) at the region of optimum stability, p_H 6·51;

(2) at an alkaline p_H, 8·82;

(3) at an acid p_H, 5·02.

The results are set out in the following tables, where the method employed for the determination of the quantity of enzyme is also stated.

Table III. *The critical increment at* p_H *6·51.*

Method of determination that of Willstätter *et al.* [1926]. Glycerol content of solution 24 %.

Time t mins.	Trypsin units per cc.		k_{uni}
	Temp. 50°.		
0	1·53		—
120	1·23		$1·82 \times 10^{-3}$
295	0·95		$1·60 \times 10^{-3}$
385	0·80		$1·68 \times 10^{-3}$
		Mean	$1·70 \times 10^{-3}$
	Temp. 60°.		
0	1·51		—
18	1·23		$1·14 \times 10^{-2}$
36	1·03		$1·06 \times 10^{-2}$
54	0·805		$1·16 \times 10^{-2}$
72	0·62		$1·24 \times 10^{-2}$
		Mean	$1·15 \times 10^{-2}$

Substituting the mean values of k_{uni} at 50° and 60° into the equation cited above we arrive at a value of $E = 40{,}800$ calories for the heat-inactivation of trypsin at p_H 6·51.

Table IV. *The critical increment at* p_H *6·50.*

Method of determination that of Linderstrøm-Lang and Steenberg [1929]. Glycerol content of solution 24 %.

Time t mins.	Trypsin units per cc.		k_{uni}
	Temp. 50°.		
0	0·72		—
90	0·60		$2\cdot01 \times 10^{-3}$
160	0·55		$1\cdot68 \times 10^{-3}$
200	0·49		$1\cdot92 \times 10^{-3}$
		Mean	$1\cdot87 \times 10^{-3}$
	Temp. 60°.		
0	0·72		—
20	0·56		$1\cdot25 \times 10^{-2}$
40	0·45		$1\cdot17 \times 10^{-2}$
60	0·35		$1\cdot20 \times 10^{-2}$
		Mean	$1\cdot21 \times 10^{-2}$

From these values of k_{uni} at 50° and 60° we arrive at a value of $E = 39{,}700$ calories for p_H 6·50.

Table V. *The critical increment at* p_H *8·2.*

Method of determination of enzyme quantity that of Willstätter *et al.* [1926]. Glycerol content of solution 24 %.

Temp. 50°. Mean value of $k_{uni} = 4\cdot35 \times 10^{-3}$
Temp. 60°. Mean value of $k_{uni} = 3\cdot47 \times 10^{-2}$

From these values of k_{uni} at 50° and 60° we arrive at a value of 44,300 calories for the critical increment at p_H 8·82.

Table VI. *The critical increment at* p_H *5·02.*

Method of determination of enzyme quantity that of Linderstrøm-Lang and Steenberg [1929].

Temp. 50°. Mean value of $k_{uni} = 2\cdot90 \times 10^{-3}$
Temp. 60°. Mean value of $k_{uni} = 1\cdot75 \times 10^{-2}$

From these values of k_{uni} at 50° and 60° we arrive at a value of 38,300 calories for the critical increment at p_H 5·02.

From these results it is seen that the critical increment of the heat-inactivation of trypsin is sensibly the same over the p_H range 5 to 9.

DISCUSSION.

It may be pointed out that the critical increment of heat-inactivation of trypsin is about one-half of the critical increment of heat-denaturation of haemoglobin and about one-third of the critical increment of heat-denaturation of egg-albumin in the region of neutrality, *i.e.* the region in which the rate of denaturation is least. If heat-inactivation be connected with denaturation of the supporting or "core" protein associated with the enzyme it must follow that the protein involved is more easily denatured than is the free protein.

Again, comparing $E = 40{,}000$ calories for heat-inactivation with $E = 14{,}000$ calories for the tryptic hydrolysis of caseinogen [Moelwyn-Hughes, Pace and Lewis, 1930] and with $E = 22{,}000$ calories for the acid hydrolysis of acetylglycine [Escolme and Lewis, 1927] it is seen that inactivation is almost certainly not hydrolysis of the protein "core" of the enzyme.

It is difficult to draw any conclusions from the fact that the stability optimum of trypsin lies between p_H 6 and 7, because of the diverse results cited in the literature about the electro-chemical nature of trypsin. Thus Michaelis and Davidsohn [1911] stated that trypsin was an amphoteric body with an isoelectric point between p_H 4 and 5, while Northrop [1924] agreed that trypsin was amphoteric, but found the isoelectric point to be at p_H 10·2. Later work by Willstätter has indicated however that it is only in crude preparations that trypsin has an amphoteric character. After purification trypsin is not adsorbed by alumina suspensions having a negative charge—but is adsorbed quantitatively by kaolin suspensions which have a positive charge. From this it appears that trypsin is definitely basic in character.

It is seen that the region of optimum stability for trypsin, p_H 6–7, does not coincide with the region of optimum activity, which is p_H 7·8–8·7. In this connection, however, it should be pointed out that the rate of destruction of trypsin in a digestion mixture maintained about p_H 8·7 will not be so great as the rate of destruction found for solutions containing enzyme alone at that p_H, because in the former case the products formed during the tryptic digestion exert a considerable stabilising influence upon the enzyme.

SUMMARY.

1. Experiments have been carried out on the heat-inactivation of trypsin which has been purified so as to be free from enterokinase and its pre-stage. The importance of this purification of an extract of pancreas is emphasised on account of the complicating effect of the "spontaneous activation" of the enzyme which otherwise occurs. It is found that the course of the heat-inactivation of trypsin purified in this way follows the unimolecular equation.

2. The effect of p_H upon the heat-inactivation of trypsin prepared in the above manner has been studied. It has been found that trypsin has an optimum stability about p_H 6·5, and that the rate of destruction increases markedly on either side of this point. The p_H-stability curve is similar in shape to that obtained for the effect of p_H upon the rate of heat-denaturation of proteins.

3. The critical increment for the heat-inactivation process has been determined (*a*) in the alkaline region, (*b*) in the acid region, (*c*) at the region of optimum stability. It is found that the critical increment is sensibly the same at all three points and is of the order of 40,000 calories per molar unit of enzyme.

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LXIX. INSULIN AND INCREASE IN WEIGHT OF YOUNG ANIMALS.

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(*Received April 15th, 1930.*)

All those who have made use of rabbits for the assay of insulin have noted that most of the animals gain rapidly in weight during the first few weeks. On the other hand, according to Long and Bischoff [1930], insulin has no influence on the growth curves of rabbits. It has also been observed that depancreatised dogs treated with insulin and maintained on a diet of meat, raw pancreas and sucrose can be made to put on fat by increasing the proportion of sucrose, allowing at the same time one extra unit of insulin for every 2·5–3 g. of sugar [Macleod, 1926]. It is also well known that most diabetic patients who are allowed a liberal diet with sufficient insulin to prevent hyperglycaemia tend to put on weight rapidly. These experiences soon suggested to clinical workers that insulin might be of value in the treatment of malnutrition, particularly of children. The first observations in this direction were those of Pitfield [1923], and they have been followed by numerous others of which we will mention only those of Marriott and of Tisdall *et al.* Marriott [1924] concluded that rapid improvement in the nutrition of non-diabetic, malnourished children usually followed the intravenous injection of glucose and insulin; but this conclusion was not endorsed by Tisdall *et al.* [1925]. In view of these findings it seemed to us of interest to ascertain whether young pigs while being fed on a good fattening diet could be made to lay on fat more rapidly than ordinarily by injections of insulin. The insulin was supplied in solid form by the British Drug Houses, Ltd., and, after being dissolved in faintly acidulated water in suitable concentrations, was assayed by the rabbit method.

EXPERIMENTAL.

Twenty-four pigs of approximately equal weights were selected at weaning and placed on the succession of four graduated diets adopted in the Pig Department of this Institute as the most suitable for raising them for market purposes. Each diet was further supplemented by skim milk. The dry feed and skim milk, as well as water, were supplied to all animals *ad libitum.* The consumption of food per group was estimated daily, by weighing the unconsumed food. The pigs were weighed every third morning before feeding and, after three weighings, twenty animals of approximately equal weights were selected and divided into two equal groups. The animals of Group I served as controls, and those of Group II were divided into two equal sub-groups (*a* and *b*) for injection with insulin.

The four pigs, which were not included in the above-mentioned groups, were used for studying the general effects of insulin. Two of them were deprived of food overnight and each was injected at about 10 a.m. with three units of insulin per kg. The blood-sugar, examined at hourly intervals in blood taken from the tail or ear, fell in the course of about three hours to below 0·05 % when hypoglycaemic symptoms developed, the characteristic features being rapid breathing with moderate hyperexcitability which was soon followed by muscular weakness and a state of coma with falling rectal temperature and marked cyanosis. In order to restore the animals, very large amounts of 50 % solution of glucose had to be injected subcutaneously. Although these injections were started as soon as the coma had become established, no improvement in the condition could be noted for nearly an hour. Recovery was then rapid in one of the animals, but very gradual in the other, both being, however, practically completely restored to normal by 5 p.m.

Other animals were injected with one unit of insulin per kg. body-weight, without restriction of food. The following figures show a typical effect on the percentage of blood-sugar.

	a.m.					p.m.			
Time	7·45	8.0	9.15	10.20	11.30	12.30	3.0	4.0	4.45
Blood-sugar mg. per 100 cc.	99	Insulin 1 unit per kg. Fed	69	60	73	83	94	Fed	121

In the light of these results it was decided to inject into each of the five pigs of Group II *a* 0·75, and into each of the five pigs of Group II *b* 1 unit of insulin per kg. body-weight twice daily, at 7 a.m. and 4 p.m.

The insulin was injected subcutaneously behind the ears, but no samples of blood were taken, since it was feared that the excitement and struggling which are unavoidable in collecting them might influence the results.

RESULTS.

There was no evidence of hypoglycaemic symptoms in any of the animals throughout the period of insulin administration, which lasted 21 days in the case of Group II *b* and 59 days in that of Group II *a*. The results are shown in Tables I and II.

Table I. *Average increase in weight, actual and percentage.*

	Initial weight	Increase in weight											
	5. xi. 28	11. xi. 28		17. xi. 28		23. xi. 28		29. xi. 28		20. xii. 28		3. i. 29	
	(lb.)	(lb.)	(%)	(lb.)	(%)	(lb.)	(%)	(lb.)	(%)	(lb.)	(%)	(lb.)	(%)
Group I*	71·8	5·5	7·7	12·4	17·3	19·4	27·0	25·3	35·2	47·8	66·6	64·2	89·4
Group II *a*†	75·4	7·3	9·7	14·8	19·6	23·0	30·5	27·6	36·6	51·5	68·3	64·7	85·8
Group II *b*†	68·1	6·6	9·7	12·9	18·9	18·5	27·2	25·2	37·0‡	46·3	68·0	57·3	84·1

* Average of 10 pigs. † Average of 5 pigs. ‡ Insulin stopped.

Table II. *Average food consumption (lb.), total and "per 1 lb." gain in weight.*

Group		5. xi. 28 to 11. xi. 28		11. xi. 28 to 17. xi. 28		17. xi. 28 to 23. xi. 28		23. xi. 28 to 29. xi. 28		29. xi. 28 to 20. xii. 28		20. xii. 28 to 3. i. 29	
		R	M	R	M	R	M	R	M	R	M	R	M
I	Total	18·6	27·9	19·6	29·4	21·6	32·3	23·2	34·8	81·7	122·5	55·8	83·8
	Per 1 lb. gain	3·2	5·1	2·8	4·3	3·1	4·6	3·9	5·9	3·6	5·4	3·4	5·1
II *a*	Total	22·6	33·9	23·6	35·4	25·1	37·7	26·5	39·8	95·6	143·4	56·2	84·3
	Per 1 lb. gain	3·1	4·6	3·1	4·7	3·1	4·6	5·8	8·6	4·0	6·0	4·2	6·4
II *b*	Total	18·0	27·0	19·9	29·9	21·7	32·6	22·9*	34·4*	84·6	126·8	56·2	84·3
	Per 1 lb. gain	2·7	4·1	3·2	4·7	3·9	5·8	3·4	5·1	4·0	6·0	5·1	7·7

R Dry food. M Milk. * Insulin stopped.

It can be seen that the average weight of the animals of Group II *a* increased decidedly more rapidly than that of the controls, especially during the first three weeks of the observation, but that ultimately the controls were well ahead of the injected animals. The animals receiving the larger doses of insulin (Group II *b*) did not at first put on weight as rapidly as those of Group II *a*, but they did so more rapidly than the controls. After discontinuance of insulin in this group, the animals fell behind the controls. These results are comparable with those obtained several years ago by Miss O'Brien and one of us (J. J. R. M.) who conducted a similar series of observations on groups of full-grown rabbits. The rabbits were all fed *ad libitum* with a diet rich in readily assimilable carbohydrate and certain of them were injected twice daily with sub-convulsive doses of insulin. During the first two or three weeks, the insulin-injected animals increased in weight more rapidly than the controls, but ultimately no difference could be detected between the two groups.

Attention is to be called to the fact that the early advantage shown by the injected animals does not necessarily mean that the insulin had endowed them with a greater power of converting available carbohydrate into fat or had led to retention of water in the tissues; it may merely have stimulated the appetite so that more food was ingested. It will be seen from Table II that this was the case at least during the earlier stages of insulin injection in the pigs of Group II *a*: the total food consumption was increased but the food consumption per pound of increase in weight was unchanged. At later stages in this group for each pound of increase in weight more food was required than in the controls, indicating that the insulin was now having the effect of diminishing, rather than increasing, the assimilative powers. The results of Group II *b* show that while insulin was given there was no influence either on the total food consumption or on the assimilative power. After insulin was discontinued, the latter seemed to be decreased, since each pound increase in weight required more food than in the controls. When the pigs were ultimately sold for butchering, no difference was noted between the injected and control animals.

Although these results show conclusively that insulin does not speed up the rate of fattening of normal pigs, it does not necessarily follow that it might not have this effect when injected into poorly thriving animals. It is well known to farmers that there are often one or more pigs in a litter which do not put on weight at the average rate, without there being any obvious pathological condition to account for the state of malnutrition.

An observation was accordingly planned in which four such underweight pigs were used, two being used as controls and two injected twice daily with one unit of insulin per kg. body-weight. The period of observation was forty-two days and the following were the results.

		Initial weight	Percentage increases in weight after 42 days
Control pigs	1	96 lb.	36·4
	2	69 lb.	23·2
Insulin pigs	1	79 lb.	44·9
	2	89·5 lb.	42·5

This indicates a decided improvement as a result of insulin, but more observations would be required before it could be concluded that injections of this hormone can with certainty convert the "runts" into normal animals.

SUMMARY.

Two groups of 5 healthy pigs were given insulin daily in subcutaneous doses of 1·5 and 2 units per kg. body-weight and their weights compared with those of 10 animals fed on the same diet but receiving no insulin.

Insulin had only a very slight accelerating effect on the rate of increase in weight, which was evidently due to a correspondingly greater consumption of food and not to any metabolism-sparing action.

Other experiments suggested that insulin had a more marked effect on marasmic pigs.

We desire to express our thanks to Mr D. W. Auchinachie, and Mr Wm Thomson for technical assistance.

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LXX. THE CHARACTERISTIC ABSORPTION OF ULTRA-VIOLET RADIATION BY CERTAIN PURINES.

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(*Received April 18th, 1930.*)

The measurement of the absorption of ultra-violet radiations by biological substances, as effected with the spectrophotometer, has been used with some success in recent years. Using this method Smith [1928] measured the ultra-violet absorption of uric acid and pointed out the close correspondence of the absorption of an ultra-filtrate of human plasma with that of a simple solution of uric acid.

In some unpublished work, the present writer has found that ultra-filtrates of certain other body fluids and tissue extracts have markedly different absorptions from that of plasma. The substances most likely to cause this absorption would seem to be purines.

It was therefore decided to establish the characteristic absorption of these substances and so obtain a basis for future investigation, both quantitative and qualitative, of body fluids. In this paper the results of this investigation are reported.

Historical.

Soret [1883] and Hartley [1905] investigated the absorption of purines in the ultra-violet region. Later Dhéré [1909] examined purines and pyrimidines and showed that they all exhibited selective absorption. He also demonstrated that uric acid shows one absorption band in alkaline solution and two bands in acid solution. These observations were not quantitative, owing to the limitations of the methods employed.

Smith [1928], using a Hilger spectrophotometer with rotating sector, was able to measure the absorption of uric acid quantitatively, and confirmed Dhéré's observations in acid and alkaline solutions. Recently Marchlewski and Wierznchowska [1929] have investigated adenine, guanine and uric acid in the same way; they failed however to observe the effect of hydrogen ion concentration on the ultra-violet absorption spectra.

METHODS.

The absorption spectra were measured with a Hilger quarter-plate spectrophotometer with rotating sector. The source of radiation was a condensed spark between tungsten-steel electrodes. A Hilger micrometer cell was used for determining whether Beer's law was followed. The thickness of the cell could be varied from 0 to 5 mm., the micrometer being graduated in 0·01 mm. and reading to 0·01 mm. with 5 % error.

Ilford process plates were used except where investigation of the spectrum of shorter wave-lengths than 2150 Å. was desired, in which case Schumann plates (Hilger) were employed.

EXPERIMENTAL.

Each purine was examined with the object of showing:

(1) whether Beer's law was followed;

(2) the effect of hydrogen ion concentration on the absorption spectra;

(3) the effect of chemical combination of the purine with a molecule which has no appreciable absorption at the same wave-length.

The purines were examined in as pure a state as possible. Guanine and adenine and their nucleotides were isolated from yeast nucleic acid by the method of Jones [1914]. Hypoxanthine was obtained from meat extract. The adenosine was a commercial product and two commercial samples of uric acid were investigated. The nitrogen content was taken as the standard of purity and recrystallisation repeated until the estimated content agreed within 1 % of the theoretical value.

(1) *Beer's law*[1].

The solvent in all cases was water.

The thickness of the cell was varied so that in the formula

$$E = \frac{1}{cd} \log \frac{I'}{I}$$

$c \times d$ was constant. The sector used was one of the short focus type which will not hold a cell of more than 1 cm. length. The experimental error of the spectrophotometric method is least at an ϵ value on the rotating sector of

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[1] Beer's law states that the absorptive power of molecules is independent of their concentration. This can be expressed graphically by

$$\log \frac{I}{I'} \times \frac{1}{cd} = \text{constant},$$

where I is the intensity of the incident beam of monochromatic light and I' the intensity of the same light after transmission through a layer of absorbing solution of thickness d and concentration c.

The ratio $\log \frac{I}{I'}$ is expressed as ϵ (the extinction coefficient) and is given by the reading on the sector. E (the specific extinction coefficient) has been taken in this paper as being the constant in the above expression. Molecular E is this constant when $d = 1$ cm. and c is the molecular weight of the substance in grams in one litre and is of importance only for purposes of comparison, having no practical meaning.

1·0 to 1·1. This results from two opposing sources of error. The lower the ϵ value on the rotating sector the higher the percentage error, due to the error of the spectrophotometer being constant over all ranges [von Halban and Eisenbrand, 1927]. On the other hand, the higher the ϵ value on the sector the more difficult is it to set the sector accurately owing to the scale divisions being logarithmically spaced and very close at high ϵ values. A mean is struck at $\epsilon = 1·0–1·1$ where the mechanical error is low and the accurate setting of the sector relatively easy. From these considerations the weakest solution was made so that with a thickness of 1 cm. ϵ had a value of 1·1.

Five dilutions were examined ranging from the weak to a saturated solution. Owing to the slight solubility of the purines in water the range is not large, the saturated solution having about 25–40 times the concentration of the weakest solution examined.

All the purines examined follow Beer's law within the limits investigated.

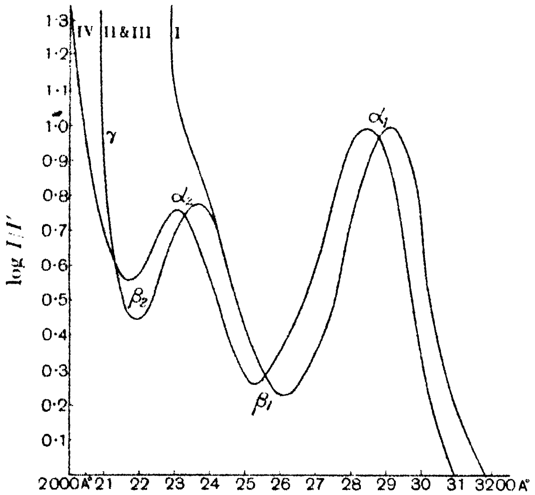

Fig. 1. Uric acid. Effect of hydrogen ion concentration on absorption curve.
I $p_H = 9·1$. II $p_H = 7·8$ III $p_H = 6·8$ IV $p_H = 2·0$

(2) *Hydrogen ion concentration.*

Owing to their general absorption at the shorter wave-lengths, buffer solutions were found unsuitable for obtaining solutions of given hydrogen ion concentration. Solutions of purines in suitable strength were made up in alkali (NaOH) and titrated with acid (HCl) to an approximate p_H. This was subsequently determined by the glass electrode method [Kerridge, 1925]. The results are shown in Tables I and II. Where the effect of hydrogen ion concentration is small, determinations at only two p_H values are given.

Absorption curves of uric acid and guanine showing the effect of hydrogen ion concentration are given in Figs. 1 and 2.

Table I.

	p_H	Molecular E		Apex/base ratio	Wave-length (Å.)		
		α apex	β base	α/β	α	β	γ
Adenine	3·0	$1·4 \times 10^5$	$0·26 \times 10^5$	5·4	2630	2300	2110
	10·0	$1·4 \times 10^5$	$0·26 \times 10^5$	5·4	2660	2380	2230
Adenine nucleotide	4·0	$1·3 \times 10^5$	$0·24 \times 10^5$	5·5	2620	2290	2110
Adenosine	8·0	$1·3 \times 10^5$	$0·24 \times 10^5$	5·5	2640	2340	2200
Hypoxanthine	3·0	$0·9 \times 10^5$	$0·15 \times 10^5$	5·6	2450	2210	2070
	7·0	$0·9 \times 10^5$	$0·15 \times 10^5$	5·6	2490	2220	2080
	11·0	$0·9 \times 10^5$	$0·15 \times 10^5$	5·6	2590	2300	2170
Caffeine	3·0	$0·8 \times 10^5$	$0·14 \times 10^5$	5·6	2740	2440	2180
	10·0	$0·8 \times 10^5$	$0·14 \times 10^5$	5·6	2710	2430	2190
Theobromine	3·0	$0·9 \times 10^5$	$0·3 \times 10^5$	3·0	2720	2410	2190
	10·0	$0·9 \times 10^5$	$0·3 \times 10^5$	3·0	2740	2480	2210
Ergothioneine	—	$1·1 \times 10^5$	—	—	2570	—	—

Table II.

	p_H	Molecular E				Wave-length (Å.)				
		α_1	β_1	α_2	β_2	α_1	β_1	α_2	β_2	γ
Uric acid	9·1	$1·0 \times 10^5$	$0·25 \times 10^5$	—	—	2920	2610	—	—	2315
	7·8	$1·0 \times 10^5$	$0·25 \times 10^5$	$0·76 \times 10^5$	$0·45 \times 10^5$	2920	2610	2360	2190	2090
	6·7	$1·0 \times 10^5$	$0·25 \times 10^5$	$0·76 \times 10^5$	$0·45 \times 10^5$	2920	2610	2360	2190	2090
	2·0	$1·0 \times 10^5$	$0·25 \times 10^5$	$0·76 \times 10^5$	$0·55 \times 10^5$	2840	2530	2300	2170	2040
Guanine	9·0	$0·63 \times 10^5$	—	—	$0·43 \times 10^5$	2760	—	—	2300	2240
	8·8	$0·63 \times 10^5$	—	—	$0·43 \times 10^5$	2760	—	—	2300	2200
	6·8	$0·63 \times 10^5$	$0·52 \times 10^5$	$0·64 \times 10^5$	$0·43 \times 10^5$	2760	2620	2500	2300	2180
	2·4	$0·63 \times 10^5$	$0·52 \times 10^5$	$0·82 \times 10^5$	$0·33 \times 10^5$	2760	2620	2500	2230	2120
	0·87	—	—	$1·09 \times 10^5$	$0·32 \times 10^5$	—	—	2480	2230	2090
Guanine nucleotide	1·0	—	—	$1·05 \times 10^5$	$0·26 \times 10^5$	—	—	2500	2230	2090

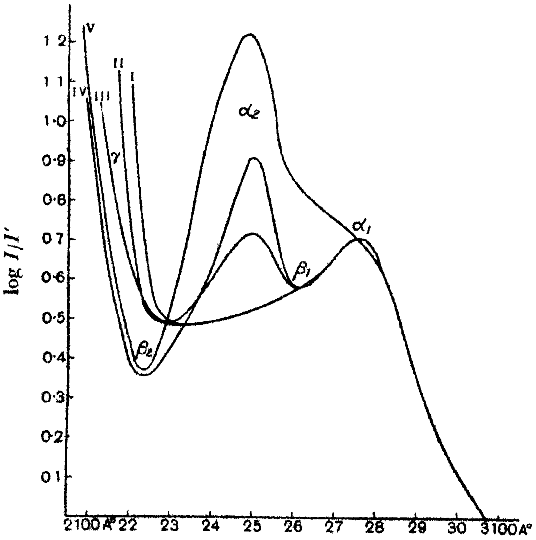

Fig. 2. Guanine. Effect of hydrogen ion concentration on absorption curve.

I $p_H = 9·0$ II $p_H = 8·8$ III $p_H = 6·8$ IV $p_H = 2·4$ V $p_H = 0·87$

Discussion of results.

It will be seen that purines can be divided into two groups: (1) those which have only one absorption band (Table I), and (2) those which have two bands in acid solution (Table II). The absorption of the first group is little influenced by increase in hydrogen ion concentration, the only effect being a slight shift of the absorption towards the shorter wave-lengths. The average shift is about 30 Å. with a change from p_H 10·0 to p_H 3·0. The exception is hypoxanthine, which shows a shift of 140 Å. over the same range of p_H. The extinction coefficient is unaffected in all cases by change of hydrogen ion concentration, which is in disagreement with Smith [1928], who found a slightly higher extinction coefficient for uric acid in alkaline than in acid solution.

The second group comprises uric acid and guanine. In the case of uric acid (Fig. 1) change of the absorption takes place in two stages: (i) between p_H 9·1–7·8 (curves I and II) and (ii) between p_H 6·8–2·0 (curves III and IV). No change occurs between p_H 7·8–6·8 (curves II and III). Two dissociation constants for uric acid [His and Paul, 1900] are given as 2×10^{-6} and $2·0 \times 10^{-9}$ which correspond to p_H values of 5·7 and 8·7 respectively. It seems probable, therefore, that in the case of uric acid the absorption changes correspond to changes between acidic and basic forms of the molecule.

In the case of guanine the change in the absorption seems to be a gradual one throughout the range of p_H. One dissociation constant is given for guanine by Wood [1903] as $8·4 \times 10^{-12}$, which corresponds to a p_H of 11·0. This is outside the range shown in Fig. 2, but a solution of guanine at that p_H shows the same absorption curve as I in Fig. 2. A titration curve of guanine showed no evidence of a second dissociation constant. From its constitution as a 2-amino-6-hydroxypurine one would expect guanine to have two dissociation constants. If, as has been suggested in the case of uric acid, absorption changes result from intramolecular rearrangements in the dissociation of guanine, it can be seen that the dissociation into the more basic form is spread over a wide range. This is probably the reason for the difficulty in determining the second dissociation constant.

The results show the importance of determining the hydrogen ion concentration in any spectrophotometric method of estimating or identifying purines.

It will be seen that chemical combinations have no effect on the absorption of the purines as judged by the absorption spectra of their nucleosides or nucleotides. The absorption of these substances is identical with that of the corresponding purines under the same conditions.

In general it may be said that the spectrophotometric method offers a fresh mode of attacking the problems of purine metabolism. It must be emphasised, however, that it can only be an adjunct to the usual biochemical methods of analysis and is useless by itself for investigating tissue fluids.

Even if the substances causing selective absorption in these fluids are purines, it can be seen from the tables that the absorption bands of the different members occur so close to one another that it would be impossible to identify and estimate two such occurring in roughly equivalent amounts. On the other hand, as a rapid method of estimating separate members of the purines the method is of great value, and the fact that nucleosides and nucleotides are estimated in terms of the contained purine might be made use of in estimating proportions of free and combined purine. In the case of uric acid, which Benedict [1923] states occurs in ox-blood combined as a riboside, the spectrophotometric method has shown, in preliminary experiments by the writer, that, by a simple method of difference, an estimate of the amount of combined uric acid can be obtained. The chemical methods only estimate free uric acid and the spectrophotometric method total uric acid, free and combined.

The chemical methods are few and of doubtful reliability. The results for uric acid in normal human blood are probably of fair accuracy, but this does not apply to animal blood [Bulmer, Hunter and Eagles, 1925]. Buell and Perkins [1928] have developed a nephelometric method for determination of adenine nucleotide in blood but, apart from these, no methods for estimating purines in small amounts of blood are available. It therefore seems reasonable to hope that the spectrophotometric method, if used with proper precautions, may be of value in investigations of purine metabolism.

It was thought of interest to include observations on ergothioneine. This compound, thiolhistidinebetaine, exists in blood and interferes with colorimetric determinations of uric acid. It was isolated from pig's blood by Hunter and Eagles [1925]. It shows selective absorption very similar to that of purines and the molecular extinction coefficient is of the same order. The interest of its absorption spectrum lies, however, in the fact that it is completely transparent to rays of short wave-length as far as 1900 Å.

SUMMARY.

1. The ultra-violet absorption spectra of the following purines has been determined: uric acid, guanine, adenine, hypoxanthine, caffeine and theobromine.

2. Beer's law is followed by all.

3. The effect of hydrogen ion concentration on the absorption spectra has been measured and its importance emphasised.

4. Combination of a purine in the form of a nucleoside or nucleotide does not alter the absorption.

5. The ultra-violet absorption spectrum of ergothioneine has also been determined.

The writer wishes to express his indebtedness to Dr J. R. Marrack for the hydrogen ion determinations, to Dr H. D. Kay for the adenine and guanine nucleotides, to Dr Eagles for the sample of ergothioneine and to Mr Campbell Smith for many valuable suggestions.

This work was done during the tenure of a scholarship given by the Worshipful Company of Grocers.

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LXXI. THE ISOLATION OF HISTAMINE FROM THE HEART.

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(*Received April 30th, 1930.*)

In connection with some problems on the physiology of the heart under investigation in this laboratory, the question arose as to whether the readily extractible depressor substance was the base histamine.

It is well known that simple aqueous or alcoholic extracts of almost any tissue cause a fall in blood-pressure on injection into the circulation of the cat. The heart is not an exception. In fact, it yields an extract with powerful depressor activity.

The marked similarity in the physiological behaviour of tissue extracts and of solutions of histamine gave rise to the view that histamine was present in these extracts, a view which has been confirmed recently by the isolation of pure histamine, in the form of its dipicrate, from extracts of liver and lung [Best, Dale, Dudley and Thorpe, 1927], of muscle [Thorpe, 1928], and of spleen [Dale and Dudley, 1929]. It therefore seemed reasonable to expect that the depressor substance of heart extracts would be histamine. This expectation has, in fact, been realised.

Apart from giving additional confirmation to the view that extracts of most tissues contain histamine, proof of the presence of histamine in heart extracts is of significance in connection with recent work on the so-called "heart hormone." Many workers have observed that heart extracts have a stimulating action on the frog's and rabbit's heart but there is much disagreement as to the nature of the substance responsible for these effects. Some workers [Demoor, 1924; Haberlandt, 1927] are of the opinion that the substance is formed in certain regions of the heart and is a specific heart hormone. Others, however [Rigler and Tieman, 1927; Rigler, 1929; Oppenheimer, 1929], deny the specificity of this substance, and have obtained extracts producing similar effects not only from any region of the heart but also from a variety of other tissues. Further, they have produced similar effects with pure histamine alone. Rigler, in fact, on physiological grounds puts forward the view that the substance is actually histamine. A full discussion of the work in this field is beyond the scope of this paper. The main features are briefly but effectively summarised by Oppenheimer [1929], who gives references to the more important papers.

The experiments described in the present communication prove that histamine is present in extracts of ox ventricles. Further, these extracts appear to contain relatively large quantities of histamine. In view of the presence of this large amount of histamine in heart extracts, it seems probable that the cardiac stimulating effect of heart extract may be due wholly or in part to histamine, and it is clear that unless histamine is removed from such extracts no conclusion can be drawn with regard to the presence of a specific heart hormone. In the absence of evidence for a specific heart hormone, chemical proof of the presence of histamine in heart extract tends to support Rigler's view.

The vasodilator activity of different tissue extracts varies considerably [Thorpe, 1928]. Lung extracts are particularly rich in histamine compared to other tissue extracts. Heart extract was found to have activity of a high order, being second only to lung extract.

Extracts prepared from four ox hearts (1·3, 1·5, 1·2, 1·7 kg.) gave values equivalent to 9·4, 18, 9·6, 8·1 mg. histamine per kg. of fresh tissue respectively when estimated by comparison with pure histamine on the blood-pressure of the cat under chloralose. The action was all of a histamine-like type and there was neither chemical nor physiological evidence to suggest the presence of a choline-like substance.

The above values apply to the ventricles of the heart. In two cases the auricles were worked up giving the interesting result that the activity, weight for weight of fresh tissue, was approximately double that of the ventricles of the heart in question. Auricle extracts therefore have nearly the same order of activity as lung extracts (40 mg. per kg.).

The technique employed for the isolation of histamine from heart extract was similar to that employed previously, alcohol being used for the extraction. In the final stages difficulties arose similar to those encountered in dealing with muscle extracts [Thorpe, 1928]. Heart muscle contains considerable quantities of creatine which during the silver fractionation is converted to creatinine and methylguanidine. The presence of these substances made the isolation of histamine picrate very difficult. The total amount of bases in the histidine and arginine fractions was small and it was found that the alcohol extraction of these could be omitted.

With regard to the freshness of the material, the hearts were obtained in the laboratory within half an hour of the animals' death at the slaughter house and the same precautions were taken to ensure sterility as in previous work.

EXPERIMENTAL.

The ventricles from a fresh ox heart (1·5 kg.) were minced and extracted for 24 hours in 3·5 l. 96 % alcohol, the extract was filtered off in a press and the meat re-extracted with a further 3 l. 60 % alcohol. The combined extracts, after addition of sulphuric acid (0·36 cc. 50 % per l.) which produced a slight precipitate, were filtered and concentrated *in vacuo* below 30° to about 250 cc.

After removal of fat by shaking with ether, the fat-free aqueous layer was concentrated to 160 cc. After removal of 2·5 g. creatine which separated on standing, the liquid was neutralised with NaOH and treated with basic lead acetate until no further precipitate was produced. The filtrate and washings from this precipitate, after removal of lead as sulphide, were concentrated *in vacuo* to 100 cc. when a further 1·65 g. creatine separated.

After removal of creatine, 50 % sulphuric acid was added to produce a concentration of 5 %. Phosphotungstic acid (25 % in 5 % sulphuric acid) was then added until precipitation was complete, 400 cc. being required. The precipitate was decomposed in the cold with baryta in the usual way, excess of baryta being removed as sulphate. The solution of bases was concentrated to 100 cc. and fractionated by the Kossel-Kutscher method. 15 cc. 40 % silver nitrate were added and the resulting precipitate ("purine" fraction) removed. Hot saturated baryta was then added cautiously until the white precipitate was just tinged with brown. The precipitate ("histidine" fraction) was filtered off. Saturation of the filtrate with baryta precipitated the "arginine" fraction. The filtrate from this precipitate forms the "lysine" fraction.

The purine and lysine fractions had no appreciable activity. Silver was removed from the histidine and arginine fractions by suspending in sufficient dilute sulphuric acid to render the liquid just acid to Congo red and treating with hydrogen sulphide. After removal of silver sulphide excess of sulphuric acid was removed by baryta and the solution of the bases from each fraction evaporated to dryness *in vacuo.*

A biological assay of the solutions before evaporation had indicated that the depressor activity of the histidine and arginine fractions was equivalent to 3·2 and 4·8 mg. histamine respectively.

The histidine fraction was treated with picric acid which immediately gave a crystalline precipitate. After a long fractionation of the picrate 0·25 g. potassium creatinine picrate and 0·1 g. creatinine picrate were separated in pure condition. The residues showed a powerful histamine-like activity but it was not found possible to isolate histamine picrate in a pure state from the creatinine picrate still remaining.

The arginine fraction contained relatively little material and was also converted to picrate. This picrate (180 mg.) was subjected to a long fractional crystallisation similar to that employed when dealing with muscle extract. The mixture consisted mainly of the picrates of methylguanidine and histamine and finally after resorting to mechanical separation of the crystals a total of 5 mg. pure histamine dipicrate was obtained. At the same time 50 mg. methylguanidine picrate were obtained.

A solution of 3·2 mg. of this histamine dipicrate in 100 cc. water had the same depressor effect on the cat's blood-pressure as a similar solution prepared from a known sample of pure histamine dipicrate. The Pauly reactions were quantitatively equal. The picrate from heart, pure histamine dipicrate and a mixture of the two all melted together at 240° with decomposition.

The yield of histamine from heart.

Throughout the purification samples were taken at the end of each stage for assaying on the cat's blood-pressure in comparison with pure histamine. The values obtained are given in Table I.

Table I.

Stage	Activity in mg. histamine
Crude extract	27
After basic lead acetate	22
Phosphotungstate	14
Purine fraction	— 0 3
Histidine fraction	— 3 2
Arginine fraction	4 8
Lysine fraction	— 0 0
Histamine isolated as pure picrate	1·0

Although rather better than that from muscle, the yield of pure histamine from heart extract is poor. But if the yields are considered at the end of each stage in the purification the losses in the earlier stages are reasonable, and comparable with those obtained in experiments with other tissues, only the loss in the silver fractionations being rather larger than usual (41 % instead of about 25 %). The yield from the picrate stage is certainly low, but it must be remembered that the experiment was performed on a very small scale. Consequently, since sufficient volume of liquid had to be left for manipulation, relatively less creatine could be removed by crystallisation in the early stages than in large-scale experiments such as those with muscle. Thus a relatively greater interference by creatinine and methylguanidine is to be expected in the picrate stage.

The physiological assays show a continuous fall in activity throughout the purification, a fall which would be expected if histamine was adsorbed by the relatively large precipitates removed during the manipulation. The losses are of the same order as in previous experiments with other tissues, and it would appear that the losses in activity are due to the removal of histamine and not to the removal of another depressor substance. This is supported by the fact that the biological assays gave no hint of the presence of any substance other than one closely resembling histamine, so that it seems justifiable to conclude that the depressor substance of heart extract prepared as described in this paper is the base histamine.

SUMMARY.

(1) Extracts of ox ventricles show a powerful depressor activity corresponding to 8 to 18 mg. histamine per kg. assayed biologically.

(2) Pure histamine, in the form of its dipicrate, has been isolated from such an extract and it is suggested that this substance is responsible for the depressor activity.

(3) The significance of these findings in relation to recent work on the so-called heart hormone is discussed.

I wish to express my gratitude to Prof. I. de Burgh Daly for carrying out a number of the physiological assays required during these experiments.

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LXXII. THE ABSORPTION AND EXCRETION OF CALCIUM AND PHOSPHORUS BY RATS RECEIVING EXCESSIVE DOSES OF IRRADIATED ERGOSTEROL.

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It has been shown by Kreitmair and Moll [1928], Harris and Moore [1928, 1929], and Kreitmair and Hintzelmann [1928], and has since been confirmed by many other workers, that the administration of excessive doses of irradiated ergosterol to animals results in the production of calcium deposits, notably in the kidney and aorta, and in a tendency to hypercalcaemia and hyperphosphataemia. There have been two theories put forward to account for the source of this extra calcium in the blood and in certain tissues. One, first suggested by Harris and Moore, postulates an increased absorption from the gut together with an increased retention. Smith and Elvove [1929] suggested diminished excretion of calcium, though they did not indicate whether this decreased elimination is by way of the gut or the kidney, or both. The second theory is that the source of hypercalcaemia and depositions is the calcium of the bone substance. This has been suggested by Hess, Weinstock and Rivkin [1929–30], Baumgartner, King and Page [1929], Light, Miller and Frey [1929] and others.

Hoyle and Buckland [1929] made a study of the urinary phosphorus in hypervitaminosis D, but did not estimate the calcium. Light, Miller and Frey [1929] have given figures for calcium and phosphorus balances, but owing to the fact that no figures for food intake, urine or faeces were published it is not possible to judge what processes were at work to produce the results obtained by them. An investigation of the urinary calcium in some detail (as was done by Hoyle and Buckland for the phosphorus) followed by total balance experiments was therefore undertaken, and is described below. Just as the work was completed a paper by Brown and Shohl [1930] was published, in which they showed a definitely decreased retention of calcium after large doses of irradiated ergosterol, and a shift in the excreted calcium from the faeces to the urine. It was felt that independent confirmation of their results was probably worth publication, especially as some new points have

I. URINARY EXCRETION IN HYPERVITAMINOSIS D.

Methods.

Calcium. Shohl and Pedley's [1922] method was used, with slight modifications. As rats' urine seems to have given trouble to other workers (see Hoyle and Buckland for a discussion of the difficulties) the final routine adopted is described in full. Urine and washings from the receiving flask, approximating to 100 cc., is treated with 10 cc. concentrated sulphuric acid and 4 g. ammonium persulphate, as described by Shohl and Pedley. As the urine is heated a large bulky precipitate separates. This precipitate also occurs, though to a lesser degree, in human urine, whereas there is no trace of it in rabbit urine. By continuing the heating for 2 or 3 hours (instead of 1 hour as recommended by Shohl and Pedley), adding more ammonium persulphate from time to time whenever the urine begins to darken in colour, the precipitate becomes much reduced in amount, but does not entirely disappear. If the calcium is precipitated in its presence the final titration with permanganate gives results which are much too high. It must, therefore, be filtered off and washed twice with small quantities of hot hydrochloric acid, the washings being added to the treated urine. This washed precipitate when dissolved in a small quantity of alkali gives no reactions when tested for urates, calcium or inorganic phosphate. The precipitation of calcium can now be carried out as described in the original method. If small amounts of calcium are present, as in normal rats' urine, it is advisable to allow 24 hours for complete precipitation.

Phosphorus. The filtrate and washings from the calcium precipitate were made up to 250 cc. and used for the colorimetric estimation of phosphate, after further suitable dilution. The amount of oxalic acid present in the final dilution is too small to interfere with the colour development. The Bell-Doisy reagents [1920] were used instead of those of Briggs.

Experimental.

Eight rats, four males and four females, were kept in Hopkins's metabolism cages, two rats in each cage. The urine from each cage was kept and analysed separately, three or four days' output being mixed together. The weights of the animals were between 100 and 168 g. The diet was identical with that used by Harris and Moore [1928]. Full synthetic diet was given for 14 days before administration of the irradiated ergosterol, which was then fed at 0·1 % level. Urine was collected for 1 week only during the preliminary period. It was found essential that the animals should be kept in the metabolism cages for a few days before collecting urine, as, until they became accustomed to the change of cage, metabolism was apt to be appreciably disturbed. After 20 days of the irradiated ergosterol administration the animals were put back on to normal synthetic diet for the "curative" period. During the course of the experiment the females showed greater loss of weight than the males; two of them died at the beginning of the "curative" period. Post-mortem examination revealed the usual lesions associated with hypervitaminosis D in an advanced form. Four of the rats had some diarrhoea during the intake of irradiated ergosterol; consequently one or two samples of urine had to be discarded as they were badly contaminated with faeces.

The results of this experiment are given in Table I.

Table I. *Urinary excretion of calcium and phosphorus (mg. per rat per day).*

	Group I					Group II			
	Females		Males			Females		Males	
No. of days	Ca	P	Ca	P	No. of days	Ca	P	Ca	P
				Preliminary period.					
4	1·3	25·7	0·8	26·5	4	0·1	21·8	0·1	26·7
4	0·3	23·9	0·7	24·3	3	0·1	30·0	0·1	28·9
				Irradiated ergosterol period.					
3	5·9	25·4	5·9	27·3	4	4·5	17·2	5·3	18·5
3	5·3	17·9	7·9	24·6	4	8·3	24·5	8·9	22·6
4	2·3	15·1	3·2	10·5	4	9·9	23·5	10·1	23·8
					4	8·8	23·7	8·9	28·3
					4	8·1	23·5	8·4	25·9
				"Curative" period.					
4	—	—	2·3	17·8	3	7·3	22·4	7·0	19·1
3	—	—	1·2	30·9	3	4·3	20·4	3·8	24·2
3	—	—	1·4	28·2	2	2·2	16·8	2·0	21·3

Discussion.

These two groups of rats agreed in showing the great increase in urinary calcium as a result of the massive doses of irradiated ergosterol; this was more marked in the second group. This group did not show the sudden decrease in the calcium excretion towards the end of the irradiated ergosterol intake. The calcium in the urine of both males and females of the second group showed a rise to a maximum occurring at about the tenth day, and then followed a slow and steady decline. The concentration of calcium rose steadily throughout the period of irradiated ergosterol administration in the case of the females, whereas in the case of the males the concentration was roughly parallel to the total daily amount of calcium excreted. There was a sharp drop in the calcium concentration about 3 days after the initiation of the "curative" period (Figs. 1 and 2). The diuresis found by Hoyle and Buckland [1929] was not shown by any of these rats. The worse physical condition of the animals in the first group may explain the drop in the urinary calcium towards the end of the irradiated ergosterol period; kidney function was probably failing rapidly, as the phosphate showed the same decline and the volume of urine passed was very small. There was no increase in phosphorus excretion produced by the hypervitaminosis. This is in agreement with Hoyle and Buckland.

It is thus evident that decreased excretion of calcium by the kidney is not responsible for the hypercalcaemia or for the calcification of tissues.

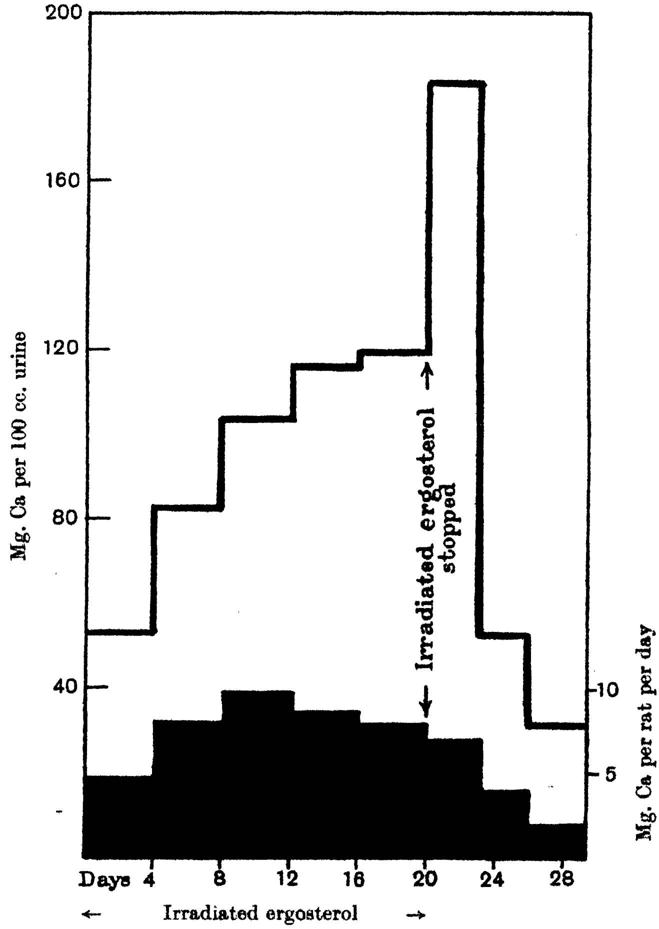

Fig. 1. ♀ Daily excretion of Ca.

II. TOTAL BALANCE EXPERIMENTS.

Methods.

The technique for the urine analyses was as described above. Calcium in the faeces was determined by a slight modification of McCrudden's method [1910] rendered necessary by the fact that there was sometimes insufficient faeces to admit of a separate phosphorus estimation. The following technique was therefore adopted in all cases to ensure uniformity. After ashing, the ash was dissolved in 5 cc. concentrated sulphuric acid, and heated with about 10 drops of perhydrol[1]. This gave a clear solution, and converted any pyrophosphate present as the result of the ashing process into orthophosphate, which could be estimated colorimetrically. The dissolved ash in the sulphuric acid was diluted to 100 cc. with distilled water, and the calcium estimated as described by McCrudden. The filtrate and washings from the calcium precipitate were made up to 250 cc. and the phosphorus determined as in the case of urine.

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[1] This method of treating the ash was suggested to me by Mr E. G. Holmes, to whom my thanks are due.

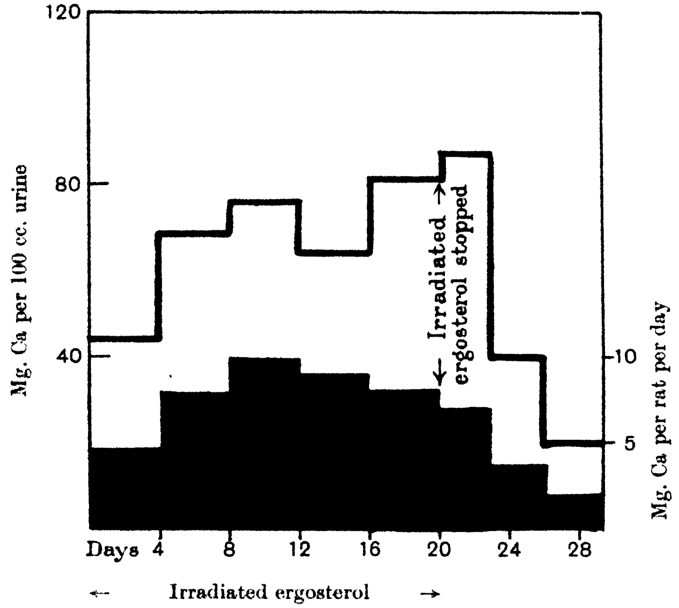

Fig. 2. ♂ Daily excretion of Ca.

Experimental.

Six albino male rats of approximately the same age were used. Each animal was kept in a separate metabolism cage, and faeces and urine were collected separately in 3 or 4 day lots. The daily amount of food fed was weighed, the dry weight of an equivalent amount determined, and any uneaten residues collected, dried and weighed. In this way the exact quantity eaten could be obtained by subtraction. Distilled water was given for drinking. The experiment as before was divided into three parts, (1) preliminary period, (2) irradiated ergosterol period and (3) "curative" period. The irradiated ergosterol was given at 0·05 % level, this avoids the complication of soft faeces or diarrhoea, yet is sufficient to cause loss of weight and pathological lesions. The weights of the animals (g.) are given below.

No. of rat	F5	F6	F7	F8	F21	F22
Beginning of irradiated ergosterol intake	135	139	159	155	160	157
End of irradiated ergosterol intake	116	123	141	142	144	143
End of "curative" period	144	150	167	164	155	160

Table II.

No. of rat	Ca intake Periods			Faecal Ca Periods			Urinary Ca Periods			Total Ca excretion Periods			Ca retention Periods		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
F5	24·2	18·0	30·1	7·2	6·9	19·8	1·5	6·6	4·9	8·7	13·5	24·7	15·5	4·5	5·4
F6	29·4	25·9	38·4	3·8	1·4	6·2	1·6	9·4	5·8	5·4	10·8	12·0	24·0	15·1	26·4
F7	33·4	21·9	36·4	9·7	6·5	21·2	2·3	7·4	3·3	12·0	13·9	24·5	21·4	8·0	11·9
F8	26·8	26·7	34·6	8·1	6·8	11·4	1·3	5·9	4·9	9·4	12·7	16·3	17·4	14·0	18·3
F21	28·6	20·2	35·2	16·7	10·1	23·9	0·1	5·3	3·6	16·8	15·4	27·5	11·8	4·8	7·7
F22	28·1	18·9	32·1	16·9	5·9	19·9	0·2	6·4	4·4	17·1	12·3	24·3	11·0	6·6	7·8

Figures represent mg. per rat per day.
Period I. Preliminary.
„ II. Irradiated ergosterol intake.
„ III. "Curative" period.

Table III.

No. of rat	P intake Periods			Faecal P Periods			Urinary P Periods			Total P excretion Periods			P retention Periods		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
F5	49·3	36·6	64·7	7·2	7·1	16·2	39·8	31·0	36·6	47·0	38·1	52·8	2·3	−1·4	11·9
F6	59·6	52·6	78·0	5·6	3·8	4·8	36·0	36·2	39·7	41·6	40·0	44·5	18·0	12·6	33·5
F7	67·9	44·7	74·0	10·4	8·5	18·4	40·4	35·2	36·4	50·8	43·7	54·8	17·1	1·0	19·2
F8	54·4	54·2	70·1	9·4	8·9	16·0	29·2	37·4	40·7	38·6	46·3	56·7	15·8	7·9	13·4
F21	58·1	41·0	71·2	11·0	10·1	23·6	28·6	38·2	39·7	39·6	48·3	63·3	18·5	−7·3	7·9
F22	57·0	37·7	65·4	12·4	5·2	19·2	29·2	28·1	36·9	41·6	33·3	56·1	15·4	4·4	9·3

Figures represent mg. per rat per day.
Period I. Preliminary.
„ II. Irradiated ergosterol intake.
„ III. "Curative" period.

The intake, output, and retention of calcium and phosphorus are given in Tables II and III. The following points are shown by an examination of the figures. (1) The urinary excretion was much the same as in the case of the rats receiving the higher dose of irradiated ergosterol. (2) The irradiated ergosterol period was characterised by a decrease (sometimes very marked) of the faecal calcium. Only two cases (F6, F22) showed a marked change in the phosphorus. (3) The urinary calcium did not return to normal in the "curative" period during the time of the experiment (9 days generally). Evidently the recovery from the effects of the hypervitaminosis is only gradual, as one would expect. (4) The faecal calcium in the recovery period tended to be very high—much higher than it was in the normal preliminary period, so that the total excretion of calcium was the highest of all in the "curative" period. The calcium intake was also highest in this period, yet even so, in two cases (F5, F7) the extra intake compared with that of the preliminary period was 6 and 3 mg. per day, respectively, while the extra output in the faeces was 12 and 11 mg. per day respectively. (5) The faecal phosphorus was definitely increased in the "curative" period in all cases except one (F6). (6) The balance of calcium was never negative when averaged for the whole period of irradiated ergosterol administration (see however later remarks on this point). In every case the balance was materially reduced by the hypervitaminosis, and in three cases was not restored to normal during the observed period of recovery. (7) The phosphorus balance was also considerably reduced by the irradiated ergosterol.

It was not felt that the average balances for the whole of the irradiated ergosterol and "curative" periods respectively gave an adequate picture of what was taking place. These balances have therefore been further analysed in Tables IV and V. Only two 3-day collections of excreta were made from rats F5 and F7, as they were in such bad condition at the end of this short time that it was not advisable to push the experiment further. Rats F6 and F8 were observed for only 6 days instead of 9 in the recovery period. In these tables also are given the so-called "percentages of absorption" of the two elements. These have been calculated tentatively as follows: the faecal excretion was subtracted from the intake and the remainder counted as the amount absorbed, and expressed as a percentage of the intake. This is not strictly accurate, because the faecal calcium consists both of calcium which has never been absorbed and of some which has been absorbed and passed back into the gut. There is no way at present of distinguishing these two fractions, so that the above method of calculating the amount absorbed is the only one possible in the circumstances.

Table IV.

No. of rat	Retention of calcium (mg. Ca per rat per day)							Calcium absorption (%)						
	I	II			III			I	II			III		
F5	15·5	10·5	-3·5	—	-1·7	13·7	1·6	70	70	34	—	33	55	7
F6	23·4	21·7	12·1	13·8	27·6	24·6	—	87	95	94	95	95	72	—
F7	21·3	17·3	-3·2	—	3·7	17·8	12·3	71	86	30	—	36	49	36
F8	17·4	18·0	11·6	9·2	22·7	12·3	—	70	80	71	68	81	45	—
F21	11·8	-8·2	11·1	5·8	-0·5	18·1	5·7	42	9	81	70	25	49	20
F22	6·5	10·9	8·4	0·6	13·1	8·2	2·4	40	66	83	57	70	50	12

Period I. Preliminary.
,, II. Irradiated ergosterol.
,, III. Recovery.

Table V.

No. of rat	Retention of phosphorus (mg. P per rat per day)							Phosphorus absorption (%)						
	I	II			III			I	II			III		
F5	2·3	6·3	-11·9	—	11·9	14·3	10·4	85	85	62	—	81	80	65
F6	18·0	14·6	7·9	14·7	29·4	38·9	—	91	94	92	93	96	92	—
F7	17·1	10·8	-11·9	—	13·8	23·1	19·5	85	91	60	—	78	75	73
F8	15·8	12·9	1·3	7·8	15·0	11·3	—	83	87	80	81	80	73	—
F21	18·5	-6·7	14·5	2·9	11·0	12·0	2·4	81	56	90	85	66	77	57
F22	15·4	10·1	8·6	-5·2	19·6	5·8	2·4	78	87	90	80	85	66	60

Period I. Preliminary.
,, II. Irradiated ergosterol.
,, III. Recovery.

It will be seen that the balance of calcium during the period of irradiated ergosterol intake may fall quickly, as in rats F5 and F7, or more steadily as in F8, or very abruptly after several days on the diet as in the case of F22. The rapidity and extent of the fall ran closely parallel to the physical condition of the animal. Two rats developed negative balances while taking the irradiated ergosterol. In most cases the amount of calcium retained was rapidly increased as soon as the excess vitamin was removed from the diet, but it is surprising to find that this improved retention was not maintained, and in five of the rats the positive balance was actually lower than normal after a few days on the normal diet. The phosphorus retention in general tended to follow similar lines.

The percentage of calcium apparently absorbed in the preliminary period was in most cases already so high that no very marked results from the irradiated ergosterol could be expected. There is however some evidence of increased absorption in all cases but one (F 5), and it is interesting to note that with rats F 21 and F 22, where the initial absorption was lower, there is very definite evidence of the action of irradiated ergosterol in this respect. But the increased absorption was not maintained, and in every instance in the recovery period there was ultimately a definitely reduced percentage of absorption. A similar mechanism occurs in the case of the phosphorus, especially during the "curative" period.

It has to be remembered that these rats were losing weight. In order to get some idea of what effect, if any, loss of weight *per se* has upon calcium and phosphorus metabolism two male rats were given full synthetic diet in gradually decreasing amounts. The loss of weight experienced was 26 and 23 g. respectively, but the animals remained in good condition. The calcium excretion by the kidney ceased entirely, but in spite of this the retention declined rapidly (Tables VI and VII). The urinary phosphorus also became reduced in amount, roughly 50 %, and the amount retained decreased, rat F 24 actually having a negative balance for the last three days. The absorption of both calcium and phosphorus was apparently much reduced by the low food intake, but again it must be emphasised that this may not be real, but may only mean that the path of excretion has been diverted from kidney to gut.

Table VI. *Calcium balance on low food intake.*

Mg. Ca per rat per day.

No. of rat	Intake	Faeces	Urine	Total	Retention	% absorption
F 23	47·6	5·3	0·9	6·2	41·4	87
	26·5	9·2	0·9	10·1	16·4	65
	17·3	6·8	Nil	6·8	10·5	60
F 24	50·8	14·2	1·1	15·3	35·5	72
	26·5	11·5	0·9	12·4	14·1	57
	17·3	9·9	Nil	9·9	7·4	43

Table VII. *Phosphorus balance on low food intake.*

Mg. P per rat per day.

No. of rat	Intake	Faeces	Urine	Total	Retention	% absorption
F 23	96·7	6·7	57·5	64·2	32·5	93
	53·9	8·8	38·2	47·0	6·9	84
	35·2	5·2	26·7	31·9	3·3	85
F 24	103·3	4·8	56·4	61·2	42·1	95
	53·9	10·3	41·7	52·0	1·9	81
	35·2	8·0	29·3	37·3	−2·1	77

DISCUSSION.

Brown and Shohl [1930] have adequately stated the difficulties and dangers attendant upon any attempt to determine from urinary and faecal examinations whether the absorption has been altered or not. The figures given above must be accepted with reserve. It would appear from them that large doses of irradiated ergosterol tend at first to increase absorption of calcium and later to decrease it. We are on surer ground when dealing with retention, and here there is no doubt that excessive doses of irradiated ergosterol, combined with the resultant low food intake and loss of weight, reduce this amount very gravely. Recovery from the deleterious effects is evidently slow, for nine days after restoration to normal diet the balances were still gravely disturbed. During this period the calcium deposits are disappearing from the body, and the fact that the urinary excretion is declining, whereas the faecal elimination is unusually high, points to the probability that much of this deposited calcium is being eliminated by the gut rather than by the kidney. Whatever the effect of irradiated ergosterol may be on absorption it is clear that the hypercalcaemia and calcification of various tissues is not due entirely, if at all, to increased absorption, since the amounts retained in the body are less than is normally the case. The source of this calcium must be looked for elsewhere, and unlikely as it appears *a priori* that the action of large doses of a substance which normally aids calcification of the bones should be in the reverse direction, yet it is difficult to see where the deposited calcium comes from if not from the bones.

The phosphorus retention was greatly reduced as the result of the irradiated ergosterol, if anything more so than was that of the calcium. Yet regarded as a whole there was less disturbance of phosphorus metabolism, for the urinary phosphorus was not increased, or at most very slightly, and there was in general a quicker settling down to normal in the "curative" period. The reduced balance during the irradiated ergosterol period must not be attributed entirely to the effect of the hypervitaminosis, for loss of weight due solely to reduced food intake produces equally severe reduction in retention.

SUMMARY.

1. Calcium and phosphorus retention in rats is decreased by excessive doses of irradiated ergosterol.

2. The urinary calcium is greatly increased, but not the phosphorus.

3. The faecal content of both calcium and phosphorus decreases, but not always in proportion to the intake; either the absorption is diminished in these cases, or increased amounts of absorbed material are being excreted by the gut.

4. Even graver disturbances in retention and absorption occur during the recovery period.

5. The low food intake and loss of weight characteristic of the hypervitaminosis probably contribute to some of these results.

6. The deposits of calcium found in certain tissues as the result of large doses of irradiated ergosterol cannot be accounted for on the grounds of increased absorption and retention.

The writer wishes to thank Dr L. J. Harris for suggesting this work and for his interest in it.

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LXXIII. THE CHEMICAL ASPECT OF THE DRYING OF TIMBER.

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INTRODUCTION.

It is common knowledge that timber does not in general find its way into commercial usage until it has attained a moisture content approaching that which will be in equilibrium with the moisture of the atmosphere in which it will be used after conversion. In view of the high moisture content of freshly felled timber, the progress of drying under natural conditions must necessarily take considerable time, but despite this fact, and the economic issues which it involves, the question often arises as to whether air-drying of timber is to be preferred to the methods of kiln-drying which are now finding widespread application in the timber-using industry. In view of the belief existing in some quarters that certain beneficial chemical changes are associated with air-drying, prejudice has arisen from time to time against the practice of drying timber in kilns.

So far as it is known, no detailed chemical investigation of the effects of the drying of wood at moderate temperatures has hitherto been carried out, although throughout the literature of wood chemistry many references are to be found regarding the effect of high temperatures. It is pointed out by Hawley and Wise [1926, p. 197] that even at temperatures far below the destructive distillation point (approximately 280°) decomposition is indicated by the formation of small traces of acetic acid and methanol, probably as a result of hydrolysis. Under the conditions obtaining in a well regulated kiln-drying treatment of timber, however, there has been some doubt as to whether chemical changes of even a minor character are to be expected, in view of the fact that Hawley [1925] has stated that no chemical difference can be detected between the results of natural air-seasoning and artificial kiln-seasoning.

Since the value of wood as a structural material is dependent largely, if not entirely, on the nature of its skeletal substance, the composition of which varies but little from species to species, and since only such physical properties as colour, odour, taste, etc., are traceable to extractives, any chemical investigation on the effect of drying must in the first instance be confined to the effects produced on the major constituents of wood substance. The presence in wood, however, of substances characterised as extractives which may play a part in the seasoning process by acting on the major constituents in some way must not be overlooked. It is for this reason, for example, that woods containing extractives of a notably acidic nature may react differently when dried under different conditions of temperature, as a certain amount of hydrolysis may be possible when high temperatures are employed. The wood of oak is known to contain small percentages of weak acids such as tannic acid, and it is conceivable that although small acid concentrations might only have a negligible effect on the major constituents of wood during air-drying, they might, none the less, have some effect when wood is dried at relatively high temperatures. As the amount of extractives in oak heartwood is invariably greater than that in the sapwood, it is conceivable that these might react differently to the same drying conditions. In the light of previous work [Hawley and Campbell, 1927] any increase in the alkali-solubility of wood after drying at relatively high temperatures would be indicative of chemical change of a hydrolytic nature. At the same time, however, there may be other chemical changes attendant on the drying of wood, which hitherto have escaped observation.

The present investigation has been undertaken therefore with a view to determining *de novo* whether any chemical changes accompany the drying of timber, and if so, whether any relationship exists between the changes attendant upon air-drying on the one hand, and artificial drying on the other.

The wood used was cut from coppice shoots of English oak of approximately 40 years' growth. After separation of the heartwood and sapwood, the material was quickly converted to sawdust and finally ground to pass an 80-mesh and be retained by a 100-mesh screen, the grinding parts of the mill being of acid-resistant steel. The analytical methods used were those recommended by Schorger [1926, p. 505] and Hawley and Wise [1926, p. 123] and the results throughout are expressed as percentages of the oven-dry weight of the original wood.

EXPERIMENTAL.

During conversion to sawdust the wood used in this investigation had undoubtedly dried to a considerable extent, but the 80–100 mesh sapwood and heartwood still retained 25·4 % and 27·2 % of moisture respectively. Moisture contents were determined both by the standard oven-drying method and the xylene method of Schwalbe [Schorger, 1926, pp. 505–506] with closely comparable results. These materials were analysed as soon as possible following conversion (Table I).

Table I. *Analysis of original wood.*

	Sapwood	Heartwood
Cold water-soluble	1·76	4·63
Hot water-soluble	4·16	9·79
Cellulose	56·46	53·01
Lignin	19·98	20·94
Methoxyl	6·36	6·92
Total pentosans	24·75	24·88
Pentosans not in cellulose	11·23	11·07
Pentosans in cellulose	13·52	13·81

Samples of both sapwood and heartwood were then extracted with 1 % NaOH solution, under the conditions laid down by Schorger [1926, p. 506] for the standard alkali-solubility determination of wood, and, after removal of the alkali by thorough washing with hot water, the residues were dried at 105° and analysed (Table II).

Table II. *Analysis of original wood after extraction with NaOH.*

	Sapwood	Heartwood
Loss on alkali extraction	19·0	22·92
Cellulose	56·96	53·87
Lignin	18·55	18·42
Methoxyl	4·70	4·23
Total pentosans	19·67	19·27
Pentosans not in cellulose	2·91	2·79
Pentosans in cellulose	16·76	16·48

The drying experiments were carried out as follows.

(i) *Air-drying.* Weighed samples of both sapwood and heartwood were placed in jars, covered with fine linen to prevent access to dust, and left in the laboratory with occasional mixing to dry for a period of 126 days. At the end of this time the moisture content of the sapwood had fallen to 13·0 % and that of the heartwood to 12·8 %.

No loss in dry weight due to air-drying was observed.

(ii) *Kiln-drying.* Weighed samples of sapwood and heartwood were subjected to a kiln-drying treatment as follows. The wood flour was in each case spread on a glass dish in a layer 1 inch deep and the following kiln-seasoning schedule, which was considered suitable for 1 inch oak boards, was applied.

Schedule employed in the kiln-seasoning of 80–100 mesh oak.

Temperature ° F.	Humidity %	Duration of treatment (days)	Total time (days)	Assumed moisture content %
115	75	2	2	30
120	65	4	6	25
125	55	5	11	20
130	45	5	16	15–10

The rate of initial warming of the sawdust was 9° F. per hour at a constant humidity of 75 %.

As a result of this treatment the moisture content of both the sapwood and heartwood fell to 7·5 %. Further, a loss of 0·2 % and 0·33 % of the dry weight of the original wood was observed in sapwood and heartwood respectively, and a decided darkening in colour was noted in each case.

(iii) *Oven-drying.* Weighed samples of sapwood and heartwood were placed in weighing bottles in a drying oven, and maintained at 105° for 200 hours. Pronounced darkening in colour took place in both sapwood and heartwood and the respective losses in weight of wood substance were 0·41 % and 1·12 %.

Analyses were now carried out on each of the residues remaining after the several drying treatments (Table III). Further, each residue was analysed after alkali extraction under the same conditions as the extractions applied to the original wood (Table IV).

Table III. *Analysis of 80–100 mesh English oak wood after kiln- and oven-drying.*

Samples		Duration of treatment (days)	Loss due to treatment	Cold water-sol.	Hot water-sol.	Cellulose	Lignin	Methoxyl	Total pentosans	Pentosans not in cellulose	Pentosans in cellulose
Air-dried	Sap	126	Nil	1·52	3·59	57·57	21·22	7·06	23·76	8·90	14·86
	Heart		Nil	4·95	9·05	54·26	22·03	7·91	23·42	8·82	14·60
Kiln-dried	Sap	16	0·20	1·81	3·65	57·27	21·98	5·81	23·74	9·44	14·31
	Heart		0·33	4·37	9·17	52·53	22·47	5·49	23·11	8·89	14·22
Oven-dried	Sap	9	0·41	2·23	4·11	55·47	21·70	5·50	23·61	11·53	12·08
	Heart		0·12	3·94	9·84	52·00	23·60	4·76	23·78	11·62	12·17

Table IV. *Analysis of air-, kiln- and oven-dried wood after alkali extraction.*

Samples		Loss due to treatment	Cellulose	Lignin	Methoxyl	Total pentosans	Pentosans not in cellulose	Pentosans in cellulose
Air-dried	Sap	18·78	57·09	18·33	4·79	20·15	4·43	15·72
	Heart	23·41	53·70	17·95	4·38	19·73	4·79	14·94
Kiln-dried	Sap	18·88	56·32	19·48	4·74	19·36	3·73	15·63
	Heart	24·62	51·94	18·51	4·10	18·62	3·04	15·58
Oven-dried	Sap	19·41	54·52	17·62	4·35	18·94	3·82	15·12
	Heart	28·93	49·30	19·60	4·10	16·52	3·42	13·10

The effect of mild hydrolysis on the original wood.

A sample of the original heartwood was subjected to a hydrolytic treatment as follows. 100 g. of the material was incorporated with 2 litres of 1 % aqueous hydrochloric acid and maintained at 100° for 6 hours. After removal of the acid, thorough washing with hot water and drying at 105°, the residue was analysed (Table V).

Table V. *Analysis of 80–100 mesh English oak wood (heart) after hydrolysis with 1 $^{\circ}/_{0}$ HCl for 6 hours at 100°.*

Loss on hydrolysis	33·43
Cellulose	42·44
Lignin	18·98
Methoxyl	4·06
Total pentosans	7·28
Pentosans not in cellulose	2·47
Pentosans in cellulose	4·81

DISCUSSION.

Comparison of Tables I and III shows that, irrespective of the method of drying, the percentage amount of water-soluble material in the wood under investigation does not vary except within the limits of experimental error. Changes during drying, however, are apparent in the cellulose, lignin and pentosans.

So far as the cellulose is concerned this constituent exhibits a slight increase in amount in the passage from the green to the air-dried condition in both sapwood and heartwood. A similar though not quite so pronounced increase is noted in the cellulose content of kiln-dried sapwood, while the cellulose content of kiln-dried heartwood is slightly lower than that of the original wood. In the case of the oven-dried material depreciation of the cellulose content is observed to take place in both sapwood and heartwood. These slight variations in cellulose content are further seen to be accompanied by concomitant variations in the percentage amounts of furfuraldehyde-yielding complexes in the wood, for, while the total pentosan content exhibits a constant decrease in amount as a result of all three drying processes, the pentosan content of the cellulose increases on air- and kiln-drying, and decreases on oven-drying. The variations in the pentosan content of the cellulose agree very closely with the variations in the cellulose content itself, except in the case of oven-drying where the loss of pentosans in the cellulose is greater than that of the cellulose, and in kiln-dried heartwood where a slight increase in the pentosan content of the cellulose is accompanied by a decrease in cellulose content. Coupled with the fact that only slight losses in weight have been observed as a result of kiln- and oven-drying, the depreciations of cellulose and of pentosans in the cellulose indicate that kiln-dried heartwood and oven-dried heartwood and sapwood have undergone a certain amount of incipient hydrolysis, decomposition being most pronounced in oven-dried heartwood.

From the analytical data it is impossible to deduce to the fullest extent the true nature of the changes which take place in the furfuraldehyde-yielding complexes during drying. It is none the less evident, however, that definite changes take place in that portion of such bodies which in green wood is not associated with the cellulose, since, whether an increase or decrease in the pentosan content of the cellulose be observed, depreciation of the total pentosan content is a consistent result of drying by all three processes. The results therefore indicate that, of that portion of the furfuraldehyde-yielding complexes which is not associated with the cellulose in the original wood, part becomes associated with the cellulose during drying, and remains associated with it provided the drying conditions are not too severe, and part is transformed into something which ceases to yield furfuraldehyde on distillation with 12 % hydrochloric acid. It is probable that this latter portion becomes associated with lignin during drying, as this constituent exhibits a consistent increase in amount as a result of all three drying processes studied. Thus the question of the relationship between the lignin and pentosans of wood is again revived. From the conflicting experimental evidence available on this subject up till 1926, Hawley and Wise [1926, pp. 70–71] are unable to draw definite conclusions, but Jonas [1928] has recently discussed numerous theories of lignin constitution, and arrives at the conclusion that lignin is a condensed furfuran product containing the stable tetrahydrofurfuran ring.

Further light on the nature of the chemical changes attendant on the drying of wood is obtained only by a comparative study of the effect of alkali on green and dried woods respectively. Comparison of Tables I and II shows the effect of 1 % sodium hydroxide at 100° on the original green wood.

It can be seen that the pentosans and lignin are the components most susceptible to alkali attack, but the outstanding fact is that cellulose appears to be but little affected by alkali until it is observed that the pentosan content of the cellulose in alkali-extracted wood is higher than that of the cellulose isolated from the original wood. The explanation of this fact is not yet apparent, but from the results of research at present in progress it would appear that an increase in the pentosan content of the cellulose after alkali-extraction is characteristic of hardwoods.

Comparison of Tables II and IV shows that the total respective alkali-solubilities of green and air-dried wood are approximately the same, but closer examination of the data reveals that, while the alkali-solubility of the pentosans not in the cellulose is much less in air-dried wood than it is in the original wood, an increase in the alkali-solubility of lignin is noted as a result of drying. This points to the conclusion that the increase in the alkali-solubility of the lignin is attributable not so much to any effect of drying on the lignin nucleus as to the original susceptibility to attack by alkali of those bodies derived from the furfuraldehyde-yielding complexes which have become associated with it during drying. This is further borne out by the analytical data, which show that after air-drying the increase in alkali-solubility of the lignin is approximately equal to the increase in the percentage of that constituent which results from the drying treatment.

As a result of kiln-drying, the pentosans not in the cellulose are slightly more susceptible to alkali attack than the corresponding components of air-dried wood. In this case also it seems probable that the increased alkali-solubility of lignin is accounted for by the additions made to it during drying.

So far as oven-drying is concerned, the low cellulose yields recorded, coupled with the depreciation of the pentosan content of the cellulose, suggest that the severity of the treatment has induced a certain amount of hydrolysis. This is borne out by the data in Tables II and IV, which indicate that the pentosans in oven-dried wood are more susceptible to attack by alkali than the corresponding components in kiln- and air-dried wood. In conjunction with the fact that the cellulose in oven-dried sapwood is more resistant to alkali than the corresponding components in oven-dried heartwood, the experimental evidence favours the conclusion that prolonged heating of green wood at 105° has the effect of inducing hydrolysis, the results of which are more marked in heartwood than in sapwood.

The ease with which green oak heartwood can be hydrolysed is demonstrated by the data in Table V. Comparison of these with the data in Table I shows that the pentosans are by far the most susceptible to hydrolysis of all the principal wood components.

CONCLUSIONS.

From the foregoing considerations it is to be concluded that the drying of oak timber is associated with definite chemical changes in the furfuraldehyde-yielding complexes of the wood substance, which are responsible in their turn for alterations in the cellulose and lignin aggregates, as determined by standard analytical methods. In this respect close agreement has been proved between the nature of the chemical changes attendant on the processes of air- and kiln-drying, although the analytical data suggest that kiln-drying may induce very slight decomposition in heartwood. The effects of drying by continuous heating of wood at 105° for 200 hours are marked by a certain amount of decomposition which has been shown to be of a hydrolytic nature. It must further be concluded that the greater amount of hydrolysis noted in the heartwood is due to its higher content of extractives of an acidic nature. In this connection it is probable that the reason why it is found in practice that softwoods are much easier to dry at elevated temperatures than hardwoods, is that softwoods invariably contain less pentosans than hardwoods. The susceptibility of the pentosans of wood to hydrolysis has been amply demonstrated in previous work, but having regard to the evidence cited above it is conceivable that conditions which would be severe enough to cause marked depletion in the pentosans of a hardwood—with possible influence on the other major constituents, and probably the physical properties of the wood as a whole—might produce no marked detectable difference on a softwood, solely on account of the low pentosan content of the latter. While it is admitted that the effects of drying on the wood flour used in this investigation cannot implicitly be claimed for wood in its natural form, owing to purely structural considerations, the results show that in so far as its effect on wood substance is concerned, a well-regulated and suitable kiln-drying treatment should have no detectable deleterious effect. It is indicated, however, that if the conditions obtaining in the interior of a piece of wood in the process of kiln-drying should by any chance become so drastic as to approach the conditions obtaining in an oven-drying treatment, deleterious effects might reasonably ensue. This is all the more probable for the reason that if volatile acids cause the hydrolysis, their action would be of a more serious nature than that detected in the present investigation, in view of the fact that they could not volatilise so readily from the interior of a piece of wood as they could from the more discrete particles of a wood flour.

SUMMARY.

1. The effects of air-, kiln- and oven-drying on both sapwood and heartwood of English oak have been examined in detail.

2. It is shown that air- and kiln-drying of wood are associated with chemical changes in the furfuraldehyde-yielding complexes of wood substance, to the extent that total pentosans are depreciated while the pentosan content of the cellulose is enhanced, and an increase in lignin is noted.

3. Oven-drying of wood is shown to result in an increase of lignin and a slight amount of hydrolysis of the carbohydrate components, which is more marked in the heartwood.

4. Reasons are given for concluding that well-regulated kiln-drying treatment of timber is strictly comparable in effect to ordinary air-drying. On the other hand, it is shown that if conditions in a kiln should become so drastic as to approach those obtaining in an oven-drying treatment, decomposition of the wood substance would be imminent.

5. It is suggested that the reason why softwoods can be kiln-dried with greater ease than hardwoods, is to be found in the fact that these invariably contain less pentosans than hardwoods, so that drying conditions which might be severe enough to hydrolyse the pentosans of wood would always affect hardwoods more than softwoods.

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LXXIV. STUDIES ON PECTIN.

V. THE HYDROLYSIS OF PECTIN.

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INTRODUCTION.

MANY workers have shown that pectin exists normally as the methyl ester of the tetrabasic pectic acid. The carboxyl groups may be, and in fact are usually, only partially esterified. The first stage in hydrolysis is the process of de-esterification which involves no change in the six-membered ring. This may be brought about in the cold by alkali as dilute as $N/20$ or by warming in weak acid solution. Heating with stronger acid or alkali results in the destruction of a portion or all of the pectin. In other words, there is a rupture of the pectic ring. A knowledge of this process may be of value in three directions. In the first place, it is of importance to know the degree of stability of the pectin molecule and the critical concentrations of various reagents above which the ring is attacked. Such conditions can then be avoided in the preparation and handling of pectic substances. In the second place, information on the rate and route of hydrolysis may throw some light on the arrangement of the units and nature of the linkages in the ring, about which so little is as yet known. Nanji, Paton and Ling [1925] suggest that the linkages are "other than 1 : 6." While it is certain that group 6 does not participate, since the carboxyl groups of the galacturonic acid units are free, it is equally certain that group 1 is involved for pectin is a non-reducing body. The amylene oxide ring theory for sugars would suggest a 1 : 4 linkage, but this has yet to be proved. Further, it must not even be assumed that the linkage between each unit is of necessity of the same type. If suitable methods of attack could be devised, any difference in type or strength of linkage would become evident from the rate of hydrolysis and nature of the products formed. In the third place, a full knowledge of the degradation products given on hydrolysis might throw much light on the down-grade processes in the metabolism of the pectic substances. There is no definite information as to the nature of the intermediate and ultimate degradation products of pectin in the plant, the metabolic picture being thus incomplete. Pectin is not a final stable substance, as is cellulose or lignin, but more labile, since it disappears during the process of lignification.

O'Dwyer [1926] found that in unlignified tissues containing pectin the hemicellulose content is low, but in lignified tissues it is considerably higher and pectin is absent or practically so. She suggested [1928], therefore, that a stage in the process of lignification is the conversion of pectin to hemicellulose. Candlin and Schryver [1928] grouped both these substances as "polyuronides," and investigated the production of hemicellulose-like bodies by the alkaline hydrolysis of pectin. They claim that the conversion is a process of decarboxylation of the uronic acid groups brought about by the action of the alkali. They quote figures for the percentage loss of uronic anhydride content of pectin treated with various concentrations of alkali under different conditions, and hold the loss of uronic acid to be an index of decomposition. However, the extent of decarboxylation of the uronic acids could only be regarded as any measure of the degree of hydrolysis of the pectin, if the uronic acid groups were the most sensitive in the molecule and were instantaneously destroyed by the alkali as the ring was ruptured. This did not seem to be in agreement with the known properties of these acids and accordingly in the work to be described the degree of hydrolysis was determined by the only valid method possible, namely by the yield of calcium pectate. For comparison, the loss of uronic acid anhydride was also estimated as in Candlin and Schryver's work. Since it was hoped at the same time to obtain information as to the relative sensitivities of the various units to alkaline treatment, and the route of the degradation, the furfuraldehyde yield of the hydrolysis liquid was also determined.

METHODS.

The methods employed were as follows.

(A) *Calcium pectate yield*; Nanji and Norman's [1928] modification of the Carré and Haynes [1922] method.

(B) *Total furfuraldehyde yield*; distillation with 12 % HCl, and precipitation of the furfuraldehyde as the phloroglucide, which is subsequently extracted with hot alcohol to remove impurities.

(C) *Total carbon dioxide yield*; Nanji, Paton and Ling's [1925] modification of the Lefèvre and Tollens [1907] method for uronic acids.

The pectin which was used throughout this work was a commercial sample prepared from Citrus fruits by the method of Wilson [1925].

The analytical figures obtained for this pectin calculated on 1 g. calcium pectate were:

furfuraldehyde yield (B)	17·72 %
carbon dioxide yield (C)	18·29 %
uronic anhydride content	73·16 %

It is seen that these figures do not precisely correspond with the theoretical. Calcium pectate should contain 69·7 % galacturonic acid anhydride, and yield approximately 19·5 % furfuraldehyde. However, commercial preparations, and, indeed, most general preparations show a divergence from these figures. The difference is accounted for in this case by a content of anhydroarabinose rather below theoretical, *i.e.* 10·33 % instead of 14·25 %, and of galacturonic acid rather higher. It is not proposed to discuss this discrepancy here except to note that the Citrus-pectin employed by Candlin and Schryver in their work gave figures differing even more widely from the theoretical. Its content of uronic acid was 88·6 % and total furfuraldehyde yield 23·1 %. Their figures are not, however, calculated on calcium pectate obtained but on the crude preparation.

Calculation of results.

Knowing the composition of the original pectin solution, it was possible from the determinations mentioned above to calculate the pentose and uronic acid content of any degradation product present in the hydrolysis liquid. The calculations involved are given below.

(D) *Furfuraldehyde due to pectin*, A × $\frac{17·7}{100}$.

(E) *Uronic acids in pectin*, A × $\frac{73·16}{100}$.

(F) *Non-pectin uronic acid*, 4C–E.

(G) *Furfuraldehyde due to non-pectin uronic acid*, F × $\frac{16·66}{100}$.

(H) *Furfuraldehyde due to pentose not in pectin* (B–D)–G.

Whereas the calcium pectate yield (A) will be the true measure of degree of hydrolysis of the pectin, the non-pectin uronic acid (F) and furfuraldehyde due to pentose groups not in pectin (H) will show the resistance or otherwise of the uronic and pentose groups to the hydrolysing agent. If either is very low or nil it will indicate that that constituent is destroyed as rapidly as it is liberated from the pectin and is, in all probability, the point at which the pectin ring is susceptible to attack.

EXPERIMENTAL.

Alkaline hydrolysis.

The work of Candlin and Schryver [1928] indicates that considerable decarboxylation of pectin is effected by boiling with alkali of concentration *N*/8. They record 33 % decarboxylation in 30 minutes and 80 % decarboxylation in 4 hours. This strength of alkali was therefore employed in the first experiments. The various hydrolyses were carried out independently for ease and rapidity of operation.

50 cc. of a 0·4 % solution of Citrus-pectin were taken in a 250 cc. round-bottomed flask, and to it were added 5 cc. *N*/10 sodium hydroxide, which was the quantity necessary to convert the pectin to sodium pectate. The solution was brought to the boiling point and 20 cc. of 1·88 % NaOH were added. This brought the volume to 75 cc. and the final concentration of alkali to 0·5 %.

The liquid was boiled under a reflux for the requisite time, rapidly cooled, and exactly neutralised with *N* HCl. In certain cases in which the hydrolysis was nearly complete double quantities had to be taken.

For the furfuraldehyde estimation and uronic acid determination the acid concentration was brought up to 12·5 % by the addition of 42 cc. concentrated HCl. In the former case the distillation was then carried out in the usual way, while in the latter a stream of CO_2-free air was passed through the cold solution for half an hour to remove any carbon dioxide in solution.

Table I. *Alkaline hydrolysis of pectin.*

Expressed on 1 g. calcium pectate.

Time	Calcium pectate yield	Loss of calcium pectate %	Total furfurald. yield	Loss of furfurald. yield %	CO_2 yield	Loss of CO_2 yield %
			Hydrolysed with 0·5 % NaOH.			
Blank	1·0	—	0·177	—	0·183	—
5 min.	0·550	45·0	0·118	33·2	0·124	32·5
10 ,,	0·256	74·4	0·052	70·8	0·098	46·7
20 ,,	0·184	81·6	0·051	71·1	0·081	55·7
30 ,,	0·122	87·8	0·043	75·9	0·073	60·1
1 hour	0·057	94·3	0·038	78·9	0·061	66·9
3 hours	0·054	94·6	0·030	83·0	0·052	71·4
			Hydrolysed with 0·2 % NaOH.			
Blank	1·0	—	0·177	—	0·183	—
10 min.	0·305	69·5	0·103	41·8	0·142	22·2
20 ,,	0·235	76·5	0·089	49·4	0·136	25·9
30 ,,	0·207	79·3	0·069	60·9	0·132	28·1
1 hour	0·202	79·8	0·059	66·7	0·113	37·9
3 hours	0·156	84·4	0·057	68·0	0·064	65·2

Since hydrolysis with 0·5 % alkali appeared to be very rapid and extensive a similar series was carried out with 0·2 % NaOH. The results obtained in both series are quoted in Table I.

All the figures are the means of two or more closely agreeing determinations.

It was clear from the above figures that the hydrolysis of pectin or, rather, the rupture of the pectin ring is more rapid than the destruction of furfuraldehyde-yielding groups and than apparent decarboxylation, and does not seem to be conditioned by either of these. It is necessary to deduce the liberation of carbohydrate units either free or in simple combination in the hydrolysis liquid. Accordingly it was of importance to determine the behaviour of sugars in the presence of alkalis under similar conditions. The sugars tested were those present in pectin, namely, arabinose and galactose. In addition, lactose was similarly treated since it contains galactose in a combined form with glucose, the last mentioned being also treated independently. 0·2 g. of sugar in 25 cc. was boiled with 5 cc. of alkali of the necessary strength to give a 0·5 % or 0·2 % solution. The solution was exactly neutralised and the reducing power measured by the iodimetric method [Baker and Hulton, 1920]. The results are expressed as percentage loss of reducing power as measured by iodine absorption (Table II).

Table II. *Treatment of sugars with alkali.*

Sugar	Conc. of NaOH %	Percentage loss of reducing power					
		2 min.	4 min.	10 min.	20 min.	30 min.	1 hour
Arabinose	0·5	44·5	49·7	51·8	—	52·8	—
,,	0·2	38·2	43·5	49·7	—	53·9	—
Galactose	0·5	41·0	41·9	45·0	—	52·8	—
,,	0·2	27·5	32·6	40·5	—	46·1	—
Glucose	0·5	31·7	34·3	40·4	—	44·5	—
Lactose	0·2	+62·4	+37·3	+21·4	+13·9	+ 9·1	+7·2

Very rapid destruction of the sugars therefore takes place in the first few minutes of treatment with dilute alkali, and the reducing power observed at 10 minutes, which thereafter remains practically constant, was thought to be due not to sugars but to iodine absorption by the degradation products formed. That this is so was shown by treating the above sugars with 0·2 % alkali for 10 minutes, cooling and neutralising with acetic acid. Phenylhydrazine was then added under the usual conditions for preparation of an osazone, but none was obtained in the cases of the monosaccharides tested or even in the case of lactose.

It seemed therefore that units with free aldehydic groups would be destroyed within a few minutes of liberation, and that any uronic acid and pentose split off from pectin by rupture of the ring and found in the hydrolysis liquid must be linked with the aldehydic group shielded. In order that the quantity of such degradation products might be determined the methods of calculation previously described were applied to the figures quoted in Table I, and are given in Table III.

Table III. *Alkaline hydrolysis of pectin.*

Expressed on 1 g. calcium pectate.

Time	Calcium pectate yield (A)	Furfurald. due to pectin (D)	Non-pectin furfurald. (B–D)	Apparent total uronic content (4 C)	Pectin uronic content (E)	Apparent non-pectin uronic content (F)	Furfurald. from non-pectin uronic acid (G)	(H)
				Hydrolysed with 0·5 % NaOH.				
Blank	1·0	0·177	—	—	0·732	—	—	—
5 min.	0·550	0·098	0·020	0·494	0·402	0·092	0·015	0·005
10 ,,	0·256	0·045	0·007	0·390	0·187	0·203	0·034	−0·027
20 ,,	0·184	0·033	0·018	0·324	0·134	0·190	0·032	−0·014
30 ,,	0·122	0·222	0·021	0·292	0·089	0·203	0·034	−0·013
1 hour	0·057	0·010	0·028	0·242	0·041	0·201	0·033	−0·005
3 hours	0·054	0·009	0·021	0·210	0·039	0·171	0·028	−0·007
				Hydrolysed with 0·2 % NaOH.				
Blank	1·0	0·177	—	—	0·732	—	—	—
10 min.	0·305	0·054	0·049	0·569	0·223	0·346	0·058	−0·009
20 ,,	0·235	0·042	0·048	0·542	0·172	0·370	0·062	−0·014
30 ,,	0·207	0·037	0·032	0·526	0·151	0·375	0·062	−0·030
1 hour	0·202	0·036	0·023	0·452	0·148	0·304	0·051	−0·028
3 hours	0·156	0·028	0·029	0·254	0·114	0·140	0·023	0·006

This method of calculation, though based on justifiable theoretical knowledge, when applied to the figures in question showed certain marked discrepancies. In the tables above, the final column, headed (H), should in theory give the furfuraldehyde due to pentose units not in pectin. It was found, however, that a negative result was obtained in practically every case. This could be due to one of two reasons. Either the total furfuraldehyde yields as obtained were uniformly low, or else the CO_2 yields were too high. It did not seem likely that any substance present in the hydrolysis liquid could lower the production of furfuraldehyde from the pentose units present on distillation with hydrochloric acid. It did, however, appear possible that the action of the alkali might produce substances, apart from uronic acids, which would give CO_2 on boiling with hydrochloric acid, although this reaction has been presumed to be specific.

The action of dilute alkalis on the polysaccharides is a field of carbohydrate chemistry as yet unexplored. The work of Lobry de Bruyn and van Ekenstein [1895, 1896, 1897] and of Nef [1907, 1910, 1914] and his co-workers on the effect of alkali on the monosaccharides has shown the very complicated nature of the reactions which take place. More recently Lloyd Evans [1929] has re-investigated these problems and extended the work to certain disaccharides. The results of all these workers have thrown much light on the constitutional relationships between the sugars. In practically all cases, however, they found it necessary to employ moderate temperatures so that the course of the reactions might be followed, and intermediary and transitory substances identified and estimated. However, differences in behaviour and quantities of end-products which were observed between individual sugars of the same series at a given alkalinity at lower temperatures were found to disappear as the temperature was raised. Evans [1929] states that at 75° the various hexose sugars yield practically identical amounts of oxidation products since they form a common 3:4-dienol. It is not unreasonable to assume that the reaction at 100° proceeds similarly to that described as taking place at 75°, but at a much increased rate. The hexoses are presumed to give in the first place an equilibrated mixture of three different dienolic forms, the 1 : 2, the 2 : 3, and the 3 : 4 isomers. Rupture then takes place at the double bonds of the several dienols and various degradation products are formed. In the cases just considered the 1 : 2-dienol gives an active pentose and hydroxymethylene, the 2 : 3-dienol, a tetrose and glycollaldehyde, and the 3 : 4-dienol, two molecules of glyceraldehyde. The pentoses and tetroses similarly give dienolic forms which on rupture yield the same products. Subsequent oxidation results in the production of CO_2 from hydroxymethylene, and in part from glycollaldehyde and glyceraldehyde. Glycollaldehyde at the same time gives oxalic acid, or may give some glycollic acid. Glyceraldehyde is converted into pyruvic aldehyde which in turn gives lactic acid or may give acetic and formic acids.

The extent to which this process is modified by linkage of the sugar is, as yet, not fully determined. The only linked hexose product which has been investigated under alkali treatment is maltose. Evans and Benoy [1930] have shown that alkaline solutions of this sugar contain an equilibrated system similar to that observed with the hexose sugars, the components of the system being the 4-glucosido-glucose dienols and their fission products. In the case of the treatment of pectin with alkali the processes of hydrolysis and dienolic fission must go on simultaneously, and upon the precise point or points of rupture of the ring will depend the course of the subsequent fission. In addition to the products given above, derivable from the hexose and pentose units, the presence of galacturonic acid renders other products likely. These it is proposed to investigate later. A 3 : 4 dienol of galacturonic acid would give one molecule of glyceraldehyde and an aldehyde acid, giving in turn probably pyruvic, glyoxylic or oxalic acid.

The possible ultimate fission products of pectin on alkaline treatment are therefore, formic, acetic, oxalic, lactic, glycollic, glyceric, glyoxylic and pyruvic acids. It seemed not unlikely that one or more of these might yield CO_2 on treatment with 12 % HCl, and its presence in the hydrolysis liquid be responsible for the unaccountably high figures for CO_2 yield obtained in the experimental work just described. Samples of each were therefore boiled with HCl under the usual conditions, and the CO_2 evolved, if any, estimated (Table IV).

Table IV. *CO_2 evolution from certain acids on treatment with 12 °/₀ HCl.*

Acid	CO_2 evolved %	Acid	CO_2 evolved %
Formic acid	Trace	Glycollic acid	Nil
Acetic acid	Trace	Glyoxylic acid	Nil
Oxalic acid	0·42	Glyceric acid	Nil
Lactic acid	0·08	Pyruvic acid	5·4

It will be seen that among these only pyruvic acid was found to yield any quantity of CO_2. It was not, however, found possible to characterise this acid in the hydrolysis liquid, owing to the presence of other decomposition products, but theoretical considerations and this indirect evidence point to its formation in this process. Oxalic acid is known to be formed in some quantity and undoubtedly must contribute to the yield of CO_2.

For comparison a hexose and a disaccharide were treated with 0·2 % alkali for various periods and after neutralisation 12 % HCl was added and the CO_2 obtained on distillation measured (Table V).

Table V. *Hydrolysis of sugars with 0·2 °/₀ alkali.*

Sugar	Time of heating min.	CO_2 yield %
Glucose	2	0·98
,,	5	0·82
,,	15	0·70
,,	30	0·73
Lactose	30	1·12

In the method of calculation described earlier, CO_2 from the dienolic fission products would be calculated as uronic acid anhydride thus giving for this, as was suspected, a high and unreliable figure. Since it is not possible satisfactorily to remove these fission products from the hydrolysis liquid, it is patent that this method of calculation is inapplicable for the purpose of throwing light on the composition of the higher degradation products of pectin.

Certain other methods of hydrolysis were tested, since it was supposed that these disadvantages might be confined to alkaline treatment. Acid hydrolysis seems to have received even less attention than hydrolysis by alkali. Ehrlich [1917] states that mild acid hydrolysis by boiling for several hours with 1 % oxalic acid yields an araban and a monobasic acid, galactose-galacturonic acid, $C_{12}H_{20}O_{12}$. Complete acid hydrolysis by boiling for 2–3 hours with 1 % HCl is stated to go further and to give free galactose and galacturonic acid. Hydrolyses were therefore carried out employing these two reagents under precisely similar conditions as described for the alkaline treatments. Since it was necessary to remove the oxalic acid before the uronic acid determinations were made, a slight excess of calcium chloride was added and the precipitated calcium oxalate filtered off. The washing of the alcohol precipitate in the pectin estimation with acid alcohol had to be carried out with great care till the oxalate test was negative. The results obtained, together with the calculations therefrom, are given in Table VI.

Table VI. *Hydrolysis of pectin.*

Expressed in g. per 1 g. calcium pectate.

Time hours	Calcium pectate yield (A)	Total furfurald. yield (B)	CO_2 yield (C)	Furfurald. due to pectin (D)	Non-pectin furfurald. (B–D)	Apparent total uronic content (4 C)	Pectin uronic content (E)	Apparent non-pectin uronic content (F)	Furfurald. from non-pectin uronic acid (G)	(H)
				Hydrolysed with 1 % HCl.						
Blank	1·0	0·177	0·183	0·177	—	—	0·732	—	—	—
1	0·549	0·255	0·210	0·097	0·158	0·840	0·401	0·439	0·073	0·085
3	0·155	0·242	0·208	0·207	0·215	0·832	0·113	0·719	0·120	0·095
5	Nil	0·231	0·128	—	0·231	0·512	—	0·512	0·085	0·146
				Hydrolysed with 1 % oxalic acid.						
Blank	1·0	0·177	0·183	0·177	—	—	0·732	—	—	—
4	0·635	0·206	0·191	0·112	0·094	0·764	0·465	0·299	0·050	0·044
8	0·325	0·282	0·190	0·058	0·224	0·760	0·238	0·522	0·087	0·137
12	Nil	0·210	0·190	—	0·210	0·760	—	0·760	0·126	0·084

An examination of these results showed two features of interest. In the first place there was observed in both cases a definite and considerable increase in total furfuraldehyde yield which can be accounted for with little difficulty. The action of the dilute acid on the galacturonic acid is in part at least a simple decarboxylation. From a unit which yields 16·66 % of its weight of furfuraldehyde is given a pentose unit which yields nearly 60 % of furfuraldehyde. The observed increase of furfuraldehyde yield is therefore due to the formation of pentose groups by simple decarboxylation of the uronic acids. In the second place an increase in carbon dioxide yield was observed in both cases despite the fact that uronic acids have undergone decarboxylation to pentoses. It is seen, therefore, that the theoretical method of calculation employed is again invalid, and it is necessary to postulate in these cases also the production of non-uronic degradation products yielding CO_2 on treatment with 12 % HCl. It is not possible to speculate on the nature of these degradation products since so little is known as to the course of mild acid scission of polysaccharides.

A final hydrolysis was carried out by heating a pectin solution alone in an autoclave at 1 atmosphere for 1 hour. Considerable acidity is developed, and the final p_H was approximately 2·0. It is this development of acidity which prevents the setting of agar media containing pectin. The results are given in Table VII. Once again an increase in total furfuraldehyde yield was observed which is only in part balanced by the loss of uronic acid, necessitating again the postulation of the production of CO_2-yielding degradation products.

Table VII. *Hydrolysis of pectin solution in autoclave at 1 atmos. (Final* p_H *= 2·0.)*

Expressed in g. per 1 g. calcium pectate.

Time	Calcium pectate yield (A)	Total furfurald. yield (B)	CO_2 yield (C)	Furfurald. due to pectin (D)	Non-pectin furfurald. (B–D)	Apparent total uronic content (4 C)	Pectin uronic content (E)	Apparent non-pectin uronic content (F)	Furfurald. from non-pectin uronic acid (G)	(H)
Blank	1·0	0·177	0·183	0·177	—	—	0·732	—	—	—
1 hour	0·904	0·219	0·172	0·160	0·059	0·688	0·687	0·001	—	0·059

Preparation of hydrolysis product.

Candlin and Schryver [1928] prepared two polyuronide products by the alkaline hydrolysis of pectin. These products both had a low uronic anhydride content, which was not surprising, since the hydrolysis was continued for 4 hours, a period which, in view of the figures given in Table I, would seem to be rather too long. Accordingly a hydrolysis was carried out for a shorter period. 20 g. of Citrus-pectin were dissolved in 1000 cc. water and NaOH added to make a concentration of 0·5 %. The whole was boiled for 1 hour under a reflux and exactly neutralised with hydrochloric acid. The solution was concentrated under reduced pressure to 200 cc. and made acid with hydrochloric acid. The precipitate which formed was filtered off, and will be referred to as the residue. The filtrate was poured into two volumes of 95 % alcohol and, after standing overnight, filtered. The precipitate was redissolved in dilute acid and reprecipitated with alcohol, and finally dried in graded strengths of alcohol. The residue was treated similarly. The yield of product was about 4 g. and of residue rather less.

The furfuraldehyde and CO_2 yields were determined on both the residue and the product.

	Residue %	Product %
Ash	6·67	16·47
CO_2 yield (ash-free)	18·27	14·31
Uronic anhydride (ash-free)	73·08	57·24
Furfurald. yield (ash-free)	17·24	14·68

It is seen that the composition of the residue closely approximates to that of the original pectin, and it is therefore unchanged pectic acid.

From the figures given above it is possible to calculate the composition of the hydrolysis product. The composition of the products obtained by Candlin and Schryver [1928] has similarly been calculated from their results, and is given for comparison in Table VIII.

Table VIII. *Composition of alkaline hydrolysis products of pectin* (%).

	(Norman and Martin) Citrus-pectin	(Candlin and Schryver) Citrus-pectin	(Candlin and Schryver) Onion-pectin
Uronic anhydride	57·24	37·25	21·55
Total furfurald.	14·68	17·40	8·43
Furfurald. due to uronic anhydride	9·53	6·21	3·59
Furfurald. from pentose	5·15	11·19	4·84
Anhydropentose	9·64	20·84	9·06
Anhydrohexose	33·12	41·91	69·39

Like the products obtained by Candlin and Schryver [1928] the hydrolysis product in this case was non-reducing, but hydrolysable by *N* H_2SO_4 into reducing substances. Arabinose was characterised by the diphenylosazone and galactose by the production of mucic acid.

GENERAL DISCUSSION.

It is not possible from the results presented above to come to any conclusions as to the arrangement of the units in the pectin molecule, or as to the type of linkage involved. Nor is one able to point to any single linkage or unit as being peculiarly susceptible to attack by a particular hydrolysing agent, and being therefore the point at which rupture of the ring takes place. A theoretically sound method of calculation for determining the composition of the polyuronide derivatives from pectin breaks down both in the case of alkaline and acid hydrolysis, owing to the production of simpler degradation products which interfere with the determination of the uronic carboxyl groups.

The susceptibility of pectin at 100° to alkali as dilute as *N*/20 is particularly noteworthy since it indicates that the carbohydrate linkages are of a type apart from those found in some other polysaccharides, such as cellulose and starch. Since the production of simple degradation products, yielding CO_2 on treatment with 12 % HCl, can be accounted for by a process of dienolic fission following upon the rupture of these linkages, it seems likely that hydrolysis follows this course. It is clear that the CO_2 yield cannot be taken as a measure of decarboxylation and still less of hydrolysis of pectin as was done by Candlin and Schryver [1928]. They quote the percentage loss of uronic anhydride calculated from CO_2 yield from pectin treated with different concentrations of alkali for various times and at several temperatures. The error in their figures due to the presence of dienolic fission products will vary with the conditions and will be considerable, as is seen in the work described above. Where it is possible to compare the apparent decarboxylation produced by the different concentrations of alkali employed by them, it appears that the lower concentrations are more effective, and in fact these workers comment on this. It seems likely that with the higher concentrations of alkali the enolic fission products are more completely degraded than with the lower, and so do not give CO_2 or as much CO_2 on treatment with HCl.

The principles underlying the mild acid hydrolysis are even more obscure, and, apart from the observation that pentose units are formed by simple decarboxylation of uronic groups, it is difficult to make further assertions. That there is simultaneously the production of non-uronic units giving CO_2 on treatment with HCl is clear from the results, but there is no evidence as to the nature of these.

It seems that valid information as to the nature of the polyuronide degradation products can only be obtained by the tedious process of actual isolation at various stages. Even then it is probable that the products isolated may be mixtures of derivatives with similar properties. That polyuronides similar in properties to the natural hemicelluloses may be obtained by the alkaline hydrolysis of pectin, as claimed by Candlin and Schryver [1928], is certain, but the process, as is shown above, is something more than simple decarboxylation, and, furthermore, can hardly be imagined as taking place in the plant. Norman [1929] has suggested that the production of hemicelluloses and of pectin in the plant is due to the mild oxidation of hexosan units. Recently Norman and Norris [1930] have shown that decarboxylation may be effected by a process of mild oxidation, thus lending some support to this theory. It is possible that enolic fission may play a part in the metabolism of these substances. Nef [1914] stated that dienols did not exist in any solution containing less than $N/30$ alkali, but this has recently been disproved by Evans *et al.* [1928] who show that limited dienol formation takes place in solutions of p_H less than 7·0. By employing copper acetate as a mild oxidant they were able to isolate some of the characteristic products of dienolic fission. It is conceivable therefore that these two methods of mild oxidation may be jointly responsible for the degradation in the plant of pectin to hemicelluloses. Oxidation of linked units may take place as in the hydrogen peroxide work of Norman and Norris [1930] and of unlinked units by limited oxidative dienolic fission owing to the p_H of the cell sap.

SUMMARY.

1. Hydrolyses of pectin have been carried out at 100° with 0·5 % and 0·2 % alkali. The yields of calcium pectate, furfuraldehyde, and CO_2 were estimated at intervals.

2. A rapid hydrolysis was observed in both cases; the rupture of the pectin ring proceeding faster than the destruction of furfuraldehyde-yielding groups and apparent decarboxylation.

3. When a calculation was made to determine the nature of the immediate degradation products of the pectin, certain discrepancies became apparent. The CO_2 yield was higher than could be accounted for from the uronic acids present.

4. On theoretical grounds the presence of certain dienolic fission products of sugars was suspected, and the CO_2 yields of these were tested. Pyruvic acid was found to yield a considerable amount of CO_2, and oxalic and lactic acids a little. There is therefore indirect evidence for their presence in the alkaline hydrolysis liquid.

5. In the course of mild acid hydrolyses an increase in furfuraldehyde yield was observed, due to simple decarboxylation of uronic acids with the production of pentose. Despite this decarboxylation, an increase of CO_2 yield was noted, owing to the formation of non-uronic degradation products of unknown composition, yielding CO_2.

6. A polyuronide derivative of pectin containing 57 % uronic anhydride was prepared by alkaline hydrolysis of pectin, and its composition was determined.

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LXXV. THE PHOSPHORUS DISTRIBUTION IN BLOOD AND THE CALCIUM AND PHOSPHORUS EXCRETION DURING HYPERVITAMINOSIS D.

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It has been shown by several workers [Harris and Moore, 1928, 1, 2; 1929; Kreitmar and Moll, 1928; Pfannenstiel, 1927] that the feeding of doses of irradiated ergosterol far in excess of the therapeutic dose produces well-marked metabolic disturbance, a condition which has been called hypervitaminosis D. This condition is characterised by an extensive deposition of calcium salts in various organs of the body and is accompanied by an increased blood-inorganic phosphate [Harris and Stewart, 1929]. Hess and Lewis [1928] also observed signs of high blood-calcium and -inorganic phosphate in clinical cases of rickets, which were receiving 2·5–5 mg. of irradiated ergosterol per day. As phosphorus occurs combined in several different forms in blood, it was thought of interest to investigate whether the increase in inorganic phosphate, which in this condition can be maintained over a long period, produces any change in the blood content of acid-soluble organic phosphorus compounds. The work reported here was carried out to investigate this point.

In these experiments, the technique of Kay and Robison [1924] has been employed, and the following four types of phosphorus compounds have been studied.

(i) The inorganic phosphate.

(ii) The acid-soluble organic phosphorus compounds, almost all of which are present in the corpuscles. They are estimated by difference between total acid-soluble phosphorus and the inorganic phosphate.

(iii) That fraction of (ii) which is hydrolysed by Robison's bone-phosphatase, "the hydrolysable fraction."

(iv) That fraction of (ii) which is not hydrolysed by bone-phosphatase, "the non-hydrolysable fraction."

These four fractions, together with the "lecithin"-phosphorus, which has not been estimated, between them contain the whole of the phosphorus in blood.

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[1] A short account of this work appeared in *Chem. Ind.* (1930), **49**, 237.

EXPERIMENTAL.

Twelve Dutch rabbits of approximately the same size and age were used in this work. At the beginning of the experiment they were about 3 months old. At the end they were fully grown and adult. The data therefore are for both growing and adult rabbits. They were given a mixed diet of bran and roots. No attempt was made either to increase or to limit the intake of calcium or phosphorus as was done in the experiments of Hess, Weinstock and Rivkin [1928]. After a control period lasting a month, the rabbits were divided into four groups and the following additions made to their diets.

A. 0·5 cc. arachis oil (2 rabbits);
B. 0·5 cc. arachis oil, containing 10 mg. irradiated ergosterol (4 rabbits);
C. 0·5 cc. arachis oil, containing 10 mg. ergosterol (2 rabbits);
D. no addition (4 rabbits).

The irradiated ergosterol used was "B.D.H.—irradiated in alcohol." The oil was fed each day by means of a pipette. The rabbits were weighed at intervals, the onset of hypervitaminosis being shown by a cessation of growth followed in time by a loss of weight.

Both during the control period and after feeding had started small samples of blood were withdrawn at intervals from the ear. The experimental feeding always followed bleeding so as not to excite the animals. The blood samples were received into paraffined crucibles, containing a minimal amount of potassium oxalate. The volume of corpuscles was estimated by haematocrit, but as it varied very little from the average figure of 34 % no corrections have been made and all results are expressed as mg. per 100 cc. of whole blood. 2 cc. were measured by pipette and delivered into 5 cc. of 25 % trichloroacetic acid. After standing for $\frac{1}{4}$–$\frac{1}{2}$ hour they were filtered into 25 cc. volumetric flasks, neutralised to phenolphthalein and made up to the mark. When not estimated immediately the filtrates were kept in the ice-chest. 5 cc. were taken for estimation of inorganic phosphate. Total acid-soluble phosphorus was estimated on 1 or 2 cc. by incineration with strong sulphuric acid and Merck's perhydrol. For the estimation of the hydrolysable fraction, 5 cc. were treated with 1 cc. of 5 % bone extract and adjusted to p_H 9. They were incubated for 24 hours (which was found to be the most suitable time) at 37° in the presence of a little chloroform. 1 cc. of N sulphuric acid was added to precipitate the proteins and, after standing overnight in the ice-chest, the precipitate was filtered off and the inorganic phosphate estimated, a correction being applied for the inorganic phosphate added in the bone extract. All estimations were carried out in duplicate by the method of Briggs [1924].

The results are shown in Fig. 1. The three types of control A, C and D, have been treated together as they behaved in the same manner. It will be seen that, although the inorganic phosphate was raised by nearly 100 % at one time when the rabbits were young, and at another by nearly 50 % when the rabbits were adult, no significant change in the content of acid-soluble organic phosphorus compounds was detected. The hydrolysable and non-hydrolysable fractions are shown in Table I. It can be seen that there are no significant differences between the control and experimental periods.

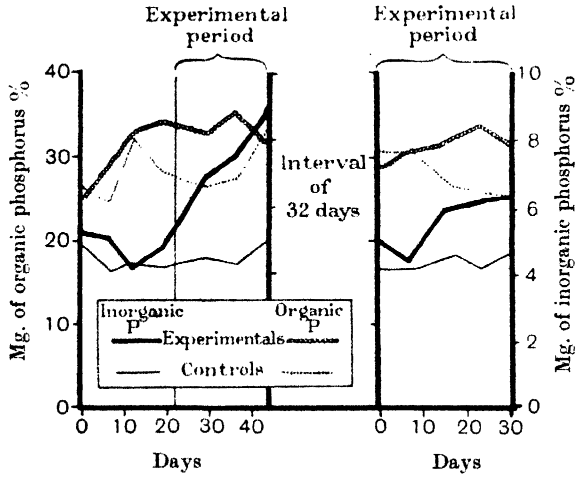

Fig. 1. Inorganic and acid-soluble organic phosphorus of control and experimental animals.

Table I. *Average values during control and experimental periods. All values mg. phosphorus per 100 cc. whole blood.*

Rabbit No.	Total organic: Control period	Total organic: Experimental period	Hydrolysable: Control period	Hydrolysable: Experimental period	Non-hydrolysable: Control period	Non-hydrolysable: Experimental period
3	33·48	31·42	6·04	4·84	27·06	26·58
4	29·44	29·80	5·80	6·15	23·64	23·65
6	32·15	32·05	5·76	5·41	26·39	26·64
7	30·82	31·90	5·80	5·67	25·02	26·13

Calcium.

It has already been mentioned that Hess and Lewis [1928] observed hypercalcaemia in clinical cases of rickets receiving 2·5–5 mg. of irradiated ergosterol per day. Hess, Weinstock and Rivkin [1928] also observed that, in adult animals with a low serum-calcium and a very low intake, the level was rapidly raised to normal by large doses of irradiated ergosterol. Harris and Stewart [1929] reported a very slight rise in the adult rabbit and a 25 % rise in the case of rats. It was therefore thought desirable to carry out some estimations of serum-calcium, and I am greatly indebted to Miss E. Watchorn for being so kind as to do these. The method used was that of Kramer and Tisdall [1921]. The results are shown in Table II. It will be seen that the experimental animals have, on the whole, a higher serum-calcium than the controls, although the control values are rather low for rabbits. It should be mentioned that the experimental animals were in a condition of hypervitaminosis during the whole period and at that time were adult. Rabbit No. 7, when killed on March 11, had a serum-calcium of 21 mg. per 100 cc. and an inorganic phosphate of about 8 mg. The symptoms were very severe and the animal had practically stopped eating and had fallen in weight from 3 lbs. to 2 lbs. in 10 days. Extensive calcification was visible to the naked eye in the aorta and kidneys, which were enlarged. Rabbit No. 6, showed the same symptoms and had a serum-calcium of 18·8 mg. per 100 cc. on March 30. It would seem that the rise in serum-calcium is a somewhat slower process than the rise in inorganic phosphate.

Table II. *Serum-calcium; mg. per 100 cc.*

	Experimental				Controls						
	Arachis oil + irradiated ergosterol				Arachis oil		Arachis oil + ergosterol		No addition		
Rabbit No.	3	4	6	7	1	2	10	12	5	8	9
Feb. 12	—	—	13·9	15·2	—	12·9	12·7	—	12·6	—	—
,, 19	14·4	13·1	—	—	—	—	—	10·1	—	10·1	10·8
Mar. 4	13·9	11·4	13·7	13·3	—	—	—	—	—	—	—
,, 11	—	—	—	21·0	—	—	—	—	—	—	—
,, 30	—	—	18·8	—	—	—	—	—	—	—	—

Table III. *Daily urinary output of calcium in mg.*

	Experimental				Controls						
	Arachis oil + irradiated ergosterol				Arachis oil		Arachis oil + ergosterol		No addition		
Rabbit No.	3	4	6	7	1	2	10	12	5	8	9
Feb. 15	37	—	—	83	—	34	Trace	—	Trace	—	—
,, 23	67	—	—	—	—	11	—	—	—	—	—
,, 24	51	—	—	29	—	—	Trace	—	Trace	—	—
,, 26	—	37	Trace	—	—	—	—	Trace	—	Trace	—
,, 27	—	—	72	—	Trace	—	—	—	—	—	—
Mar. 1	—	45	161	—	Trace	—	—	Trace	—	Trace	—
,, 3	—	108	85	—	Trace	—	—	Trace	—	Trace	—
,, 6	—	83	39	—	Trace	—	—	Trace	—	19	—
,, 7	—	130	—	—	—	—	—	16	—	53	—

Urinary calcium and phosphate.

During the latter part of the experiment urine was collected from control and experimental animals. The first fact which emerges is the absence of the diuresis which Hoyle and Buckland [1929] reported with rats. Fig. 1 shows that, if anything, the daily volumes are slightly less, which is probably accounted for by the decreased intake of food and hence water. Fig. 2 shows that the daily excretion of inorganic phosphate (estimated by the uranium acetate titration method) is consistently higher than in the controls. The urines were very alkaline (about p_H 8·5) as were also those of the controls, but, unlike the latter, they contained a white precipitate of calcium salts. Calcium estimations were again carried out by Miss Watchorn. The results (Table III) show a large excretion of calcium salts by the kidney, from a mere trace in the case of the controls to as much as 100 mg. per day in the experimental animals, which were in a condition of hypervitaminosis during the whole period. It is interesting that a sample of urine (about 25 cc.) taken from the bladder of one of the animals after killing contained 156 mg. of calcium per 100 cc.

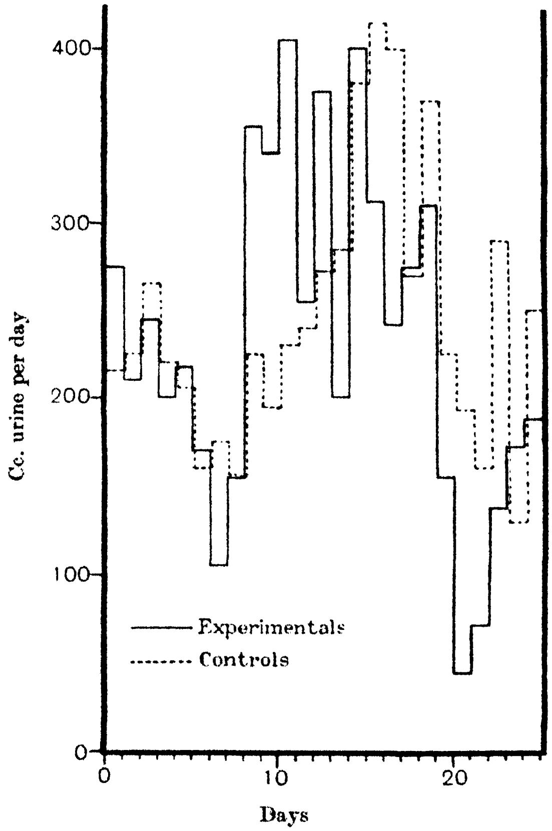

Fig. 2. Daily urinary volumes of control and experimental animals.

Recovery from hypervitaminosis.

Feeding of irradiated ergosterol to one animal was discontinued on February 26. A fortnight later, however, this animal was still excreting large amounts of calcium and a high level of inorganic phosphate by the kidney. Even on April 2 the urine contained much calcium and phosphate. When the animal was killed and examined on this date, calcification in the aorta was entirely absent and the kidneys appeared normal. It would appear from the urine, then, that recovery is very much slower than the rapidly falling blood-inorganic phosphate would lead one to suppose. On the other hand, the serum-calcium was still high, 16·6 mg. per 100 cc., while the blood-inorganic phosphate was 4 mg. per 100 cc.

DISCUSSION.

It was shown by Wigglesworth and Woodrow [1923], that, when the blood-inorganic phosphate was raised by ingestion of phosphate, no increase in the organic esters took place. On the other hand, Haldane, Wigglesworth and Woodrow [1924] and Kay [1924] observed a rise in blood-inorganic phosphate in acidosis, accompanied by a fall in the content of organic acid-soluble phosphorus compounds, which Kay traced to the non-hydrolysable fraction. He suggested that this fall was necessary to help to neutralise the free acid, and there was an increased phosphate excretion by the kidney. Conversely, alkalosis caused a diminution in free phosphate. In the experiments reported here the p_H of the plasma was not determined, but there was no sign of ketosis in the urine, in spite of the increased phosphate excretion by the kidney. It seems unlikely that the relationship between the inorganic and organic phosphorus compounds of blood can be a simple one, because, if such were the case, a change in one, unaccompanied by reaction changes, should produce a change in the other. A probable explanation of the findings reported here is to be found in the work of Jost [1927] and of Martland, Hansmann and Robison [1924]. Jost showed that by far the greater portion of the acid-soluble organic esters of the red corpuscles consists of a diphosphoglyceric

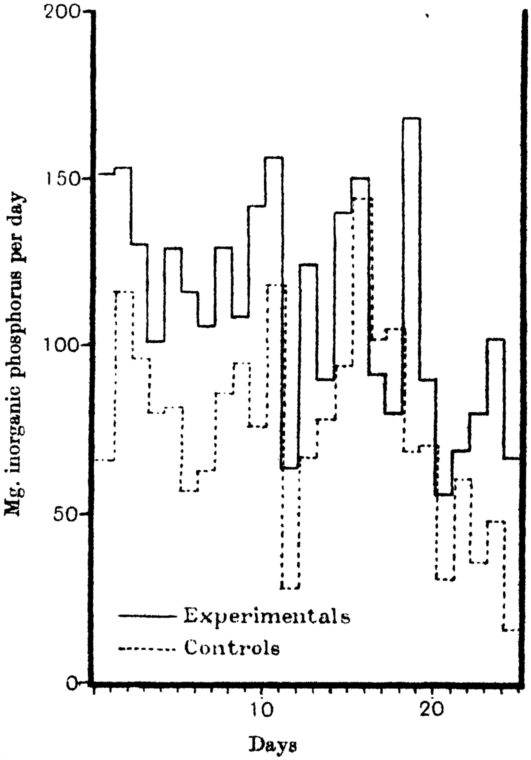

Fig. 3. Total daily urinary excretion of inorganic phosphorus by control and experimental animals.

acid, and that glycolysis in blood is associated with a synthesis of this ester. Martland, Hansmann and Robison showed the presence of an esterase in blood which attacks the organic esters under suitable conditions of p_H. Both these processes appear to be independent of the inorganic phosphate level within the limits obtaining in the blood, and hence no change in the organic esters was detected in the experiments described here. The system, in fact, is somewhat analogous to that obtaining in the synthesis and hydrolysis of hexosephosphate in alcoholic fermentation. Any accumulation of organic phosphorus esters in the plasma is probably prevented by hydrolysis in the kidney, which Eichholtz, Robison and Brull [1925] showed to be able rapidly to break down added esters.

With regard to the origin of the increase in blood-inorganic phosphate and calcium in hypervitaminosis there is no certain knowledge. It has been suggested that one of the functions of vitamin D is to regulate absorption from the gut, and the increased phosphate and calcium excretion by the kidney might suggest that this is the case. On the other hand, Hess, Weinstock and Rivkin [1929] suggest that the calcium comes from the bones. Total balance experiments in rats carried out by Light, Miller and Frey [1929], Brown and Shohl [1930] and Watchorn [1930] seem to support this view. Similar experiments under different levels of calcium and phosphate, which are being carried out in this laboratory at the present time, may serve to throw additional light on the two theories outlined above. In the experiments reported here, however, histological examination of the bones by Mr J. R. M. Innes hardly suggested that the calcium came from the bones, as these were hard and well calcified.

SUMMARY.

1. The increase in blood-inorganic phosphate, which accompanies hypervitaminosis D, does not lead to any change in the content of organic acid-soluble phosphorus compounds.

2. A rise in serum-calcium during hypervitaminosis is confirmed.

3. Hypervitaminosis produces an increase in inorganic phosphate excretion by the kidney, probably with a slight decrease in the daily urinary volume.

4. There is a greatly increased excretion of calcium by the kidney.

5. Animals recovering from hypervitaminosis D maintain a high phosphate and calcium excretion by the kidney, when, apparently, other symptoms have disappeared.

My thanks are due to Mr J. B. S. Haldane and to Dr L. J. Harris for their interest and advice in this work, and to Miss E. Watchorn for carrying out the calcium estimations, and also for much advice. The animals were in the care of Mr F. Johnson, to whom I am extremely grateful.

This work was done during the tenure of a grant from the Department of Scientific and Industrial Research, for which my thanks are due.

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LXXVI. OXIDATION-REDUCTION POTENTIALS OF CULTURES OF *C. DIPHTHERIAE*. I.

By LESLIE FRANK HEWITT.

From the Belmont Laboratories (L.C.C.), Sutton, Surrey.

(Received May 1st, 1930.)

Many bacteria, particularly facultative anaerobes, may be cultivated under widely different environmental conditions. It seems probable that the varying degrees of access to atmospheric oxygen of cultures must affect the metabolism of organisms and their general biological behaviour.

The bleaching of methylene blue is a familiar example of the reducing conditions developed in bacterial cultures during growth, but the disadvantages and uncertainties attaching to the use of dyes previously mentioned [Hewitt, 1930] necessitate the use of a more accurate method of following oxidation-reduction processes in bacterial cultures.

Reducing conditions established during growth may be followed, therefore, by the fall in potential of bacterial cultures. This has been observed by Gillespie [1921], Cannan, Cohen and Clark [1926] and Coulter and Isaacs [1929]. Fildes [1929] found that a limiting reducing potential was necessary for the germination of tetanus spores. In a previous communication [Hewitt, 1930] it has been shown that conditions of oxygen supply have a very marked effect on the potentials developed in cultures of haemolytic streptococci.

C. diphtheriae may be cultivated on the surface of solid media with free access to oxygen, in liquid media with a poorer supply of oxygen, or even anaerobically. The conditions governing toxin production are not understood. The organisms may show good growth but yield very little toxin. It is possible that such functions may be dependent on oxidation-reduction conditions in the medium, and this communication deals with observations of electrode potentials developed in cultures of *C. diphtheriae* under different conditions, with a view to possible correlation with the biological functions of the organisms.

METHODS.

The apparatus and methods used were the same as those previously described in the study of haemolytic streptococci [Hewitt, 1930].

8 cc. of culture medium were introduced into each cell, the inoculum was a small loopful of the surface pellicle of a 24-hour broth culture of Park-Williams 8 strain of *C. diphtheriae* and the incubator temperature was 37°.

RESULTS.

Peptone infusion broth (Fig. 1). Horse-flesh infusion broth containing 2 % of Difco peptone was used. The potential fell to $E_h = -0{\cdot}14$ v. during the first 10 hours' incubation and then more slowly, reaching a minimum value of just below $-0{\cdot}20$ v. in 75 hours. Even after 140 hours' incubation the potential remained below $-0{\cdot}15$ v. The potential-time curve obtained may be contrasted with that of an aerobic culture in the same broth of haemolytic streptococci. With the latter organisms the potential falls rapidly to a minimum value of $-0{\cdot}17$ v. and then rapidly rises.

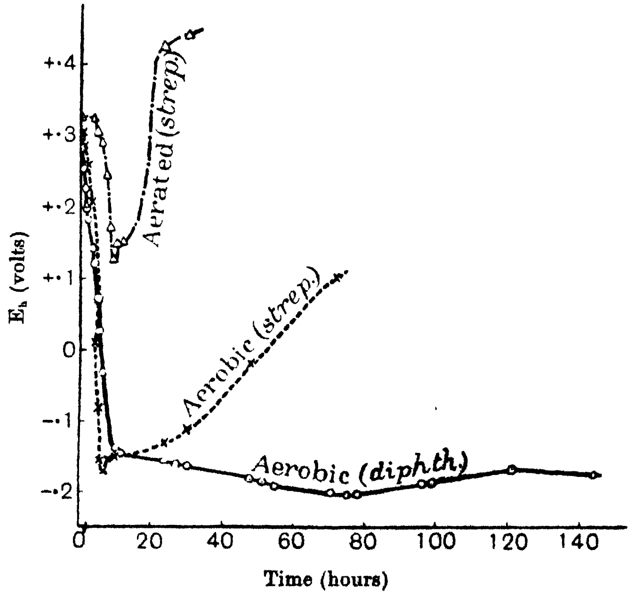

Fig. 1. Peptone infusion broth. *C. diphtheriae* and haemolytic streptococci.

Freshly heated broth (Fig. 2). Peptone infusion broth in tubes was immersed in a boiling water-bath for 30 minutes and cooled immediately before use. In the case of haemolytic streptococci the potentials of cultures in freshly heated broth fell twice as quickly and to a lower level than in untreated broth [Hewitt, 1930]. No appreciable difference, however, was observed between the potential-time curves of cultures of *C. diphtheriae* in heated and unheated broth.

Tryptic digest broth (Fig. 2). *C. diphtheriae* frequently requires acclimatisation to fresh cultural conditions. When, therefore, an inoculum was made from peptone infusion broth cultures to tryptic digest broth, growth was very slow and poor and, in conformity with this, the potential fell extremely slowly. Even after 96 hours' incubation the potential had barely reached the value $E_h = -0{\cdot}05$ v.

Sealed cultures (Fig. 3). In cultures sealed with vaselin to exclude air the potential fell extremely slowly and did not attain the low level reached in aerobic cultures. This indicates the necessity of the presence of oxygen to enable the organisms to develop intense reducing conditions. In the case of haemolytic streptococci also, access to air was found necessary for the rapid development of intense reducing conditions.

Aerated cultures (Fig. 3). When broth cultures of *C. diphtheriae* were aerated vigorously and continuously in a three-limbed cell by means of Ridley's [1928] mixing apparatus, growth was more luxuriant than in stationary cultures and was distributed throughout the medium and not confined to a surface pellicle. The potential fell to within 50 mv. of the minimum value reached in stationary cultures and then rose fairly slowly towards the level of the original broth. These results are entirely different from those obtained with haemolytic streptococci (indicated in Fig. 1).

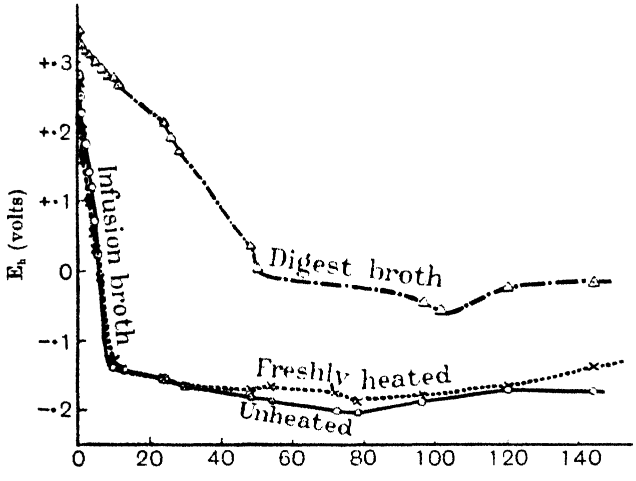

Fig. 2. Aerobic cultures of *C. diphtheriae.*

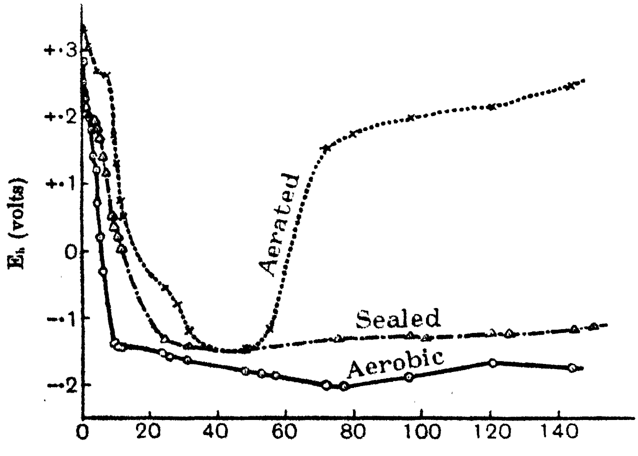

Fig. 3. Peptone infusion broth. Cultures of *C. diphtheriae.*

Table I. *Electrode potential changes in peptone infusion broth.*

Organism	Culture	Maximum fall in potential (mv.)
Streptococci	Stationary	480
,,	Aerated	200
C. diphtheriae	Stationary	500
,,	Aerated	480

With streptococci the fall in potential of aerated cultures was some 40 % of that in stationary aerobic cultures, whilst with *C. diphtheriae* the fall in aerated cultures was fully 90 % of that in stationary aerobic cultures.

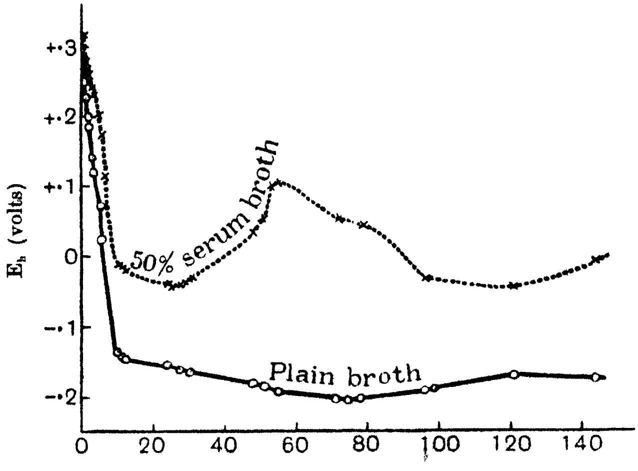

Fig. 4. *C. diphtheriae* cultures.

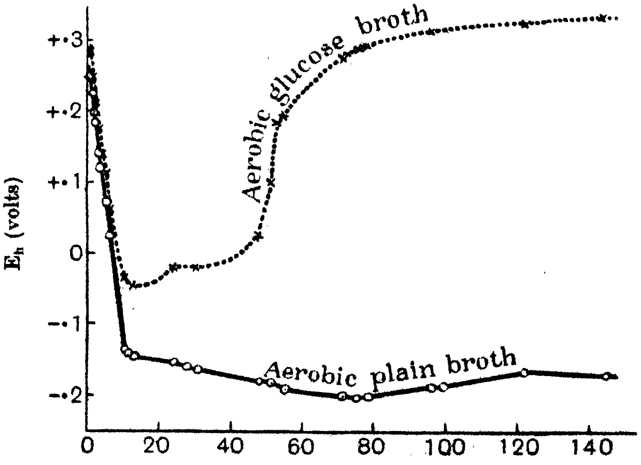

Fig. 5. Infusion broth cultures of *C. diphtheriae*.

After the phase of active growth the potential of streptococcal cultures rises rapidly to a high level, whilst the rise in potential of diphtheria cultures is relatively slow and a high (oxidising) potential is not reached.

Serum broth (Fig. 4). In 50 % serum broth the potential fell much less than in plain broth, as was observed with haemolytic streptococci.

Growth of *C. diphtheriae* appeared to cease much sooner in the presence of serum, but there is some evidence of a second fall in potential after some 60 hours' incubation.

Glucose broth (Fig. 5). For the first two days of incubation active proliferation occurred in 1 % glucose broth, but then ceased owing to accumulation of acid products. The potential did not fall to as low a level as in plain broth. This again is in conformity with results obtained with haemolytic streptococci.

Succinate broth (Fig. 6). Cultures of *C. diphtheriae* in broth containing 1 % of sodium succinate developed a deep yellow-brown colour and became very alkaline owing to oxidation of the succinate to carbonate. The surface pellicle assumed a corrugated shrunken appearance. The level of potential reached was not quite as low as in plain broth cultures and the potential began to rise after 4 days' incubation, possibly owing to the effect of the alkaline reaction on the organisms. In aerated succinate cultures also the brown colour and alkaline reaction were noted, and the potential did not fall to as low a value as in aerated cultures in plain broth.

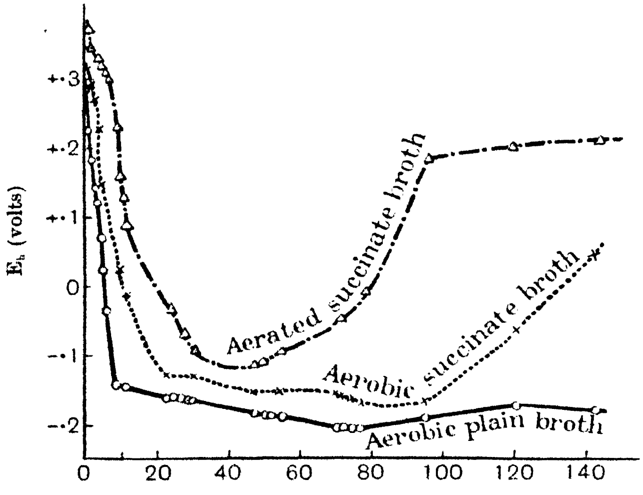

Fig. 6. Infusion broth cultures of *C. diphtheriae*.

DISCUSSION.

In general the potential-time curves of cultures of *C. diphtheriae* are less susceptible to changes in cultural conditions than are those of cultures of haemolytic streptococci. This is probably due to the intense reducing ability of *C. diphtheriae*, which appears to multiply most readily when the oxygen supply is most abundant. As with haemolytic streptococci, the presence of oxygen appears to be necessary for the rapid development of intense reducing conditions. In cultures sealed with vaselin to prevent access to air the potential falls very slowly. In aerated cultures, in which the oxygen supply is very abundant, growth is luxuriant and the potential falls to very nearly the same low level as in stationary aerobic cultures—the organisms being able easily to deal with the abundant oxygen supply and maintain reducing conditions in the medium until cessation of active proliferation.

The phase of active proliferation of *C. diphtheriae* is longer than that of streptococci and the lowest level of potential is reached more slowly, but the most marked distinction between the behaviour of the organisms is seen after the logarithmic phase of growth. With streptococci after 6–12 hours' incubation the potential begins to rise, but with *C. diphtheriae* no appreciable rise of potential is observed even after 140 hours' incubation. In aerated cultures the high level of potential corresponding to peroxide formation is rapidly attained in streptococcal cultures, but with *C. diphtheriae* this high level is not attained and the potentials do not exceed that of the original uninoculated broth. In the lack of oxidising function *C. diphtheriae* cultures are quite different from those of haemolytic streptococci, and this may be due entirely to the lack of ability to form peroxide.

The presence of serum prevents the potential of cultures of *C. diphtheriae* from falling to the low levels reached in broth. This oxygen-carrying effect, which was attributed tentatively to —S . S— groups in serum-proteins, is therefore not confined to cultures of haemolytic streptococci. Growth in serum broth was not luxuriant with *C. diphtheriae* as with haemolytic streptococci.

The effect of glucose on cultures of *C. diphtheriae* was similar to that on haemolytic streptococci, the acidity produced curtailing growth, but sodium succinate was utilised more readily by *C. diphtheriae* and on oxidation to carbonate produced an alkaline reaction which caused an early rise in potential.

The chief differences between the potentials of cultures of *C. diphtheriae* and haemolytic streptococci have been summarised in the following table. In place of the electrode potential, results are given in terms of the convenient numerical function r_H, which is the logarithm of the reciprocal of the partial pressure of hydrogen which is in equilibrium with the system. Oxidising systems have high r_H and reducing systems low r_H.

Table II. *Approximate values of* r_H *reached in cultures of* C. diphtheriae *and haemolytic streptococci.*

		r_H after					
Culture medium	Organism	6 hrs.	12 hrs.	24 hrs.	48 hrs.	72 hrs.	144 hrs.
Unboiled infusion broth ...	*C. diphtheriae*	15	9	8·5	8	7	8
,, ,, ...	Streptococci	18	10·5	14	—	—	—
Freshly heated infusion broth	*C. diphtheriae*	15	9	8·5	8	7	8
,, ,, ,,	Streptococci	9	9	9·5	13·5	18	—
Aerated infusion broth ...	*C. diphtheriae*	22·5	15·5	13	9	21	21
,, ,, ...	Streptococci	20·5	18	30	30	—	—

SUMMARY.

1. More highly reducing conditions are developed in cultures of *C. diphtheriae* than with haemolytic streptococci, especially when the oxygen supply is most abundant.

2. After the logarithmic phase of growth the electrode potentials of cultures of *C. diphtheriae* do not rise rapidly, possibly owing to the lack of a peroxide-forming function.

3. The potential-time curves of cultures of *C. diphtheriae* are less susceptible to variation with cultural conditions than those of haemolytic streptococci, probably owing to the facts stated in the first two paragraphs.

4. Serum possesses an oxygen-carrying effect in cultures of *C. diphtheriae*.

The author is deeply indebted to Dr R. G. White and Dr E. W. Todd for their interest and encouragement.

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LXXVII. OXIDATION-REDUCTION POTENTIALS OF STAPHYLOCOCCAL CULTURES. I.

BY LESLIE FRANK HEWITT.

From the Belmont Laboratories (L.C.C.), Sutton, Surrey.

(*Received May 2nd, 1930.*)

RESULTS obtained with regard to the oxidation-reduction potentials of cultures of haemolytic streptococci [Hewitt, 1930, 1] and *C. diphtheriae* [Hewitt, 1930, 2] have been reported already. Close similarities in some respects and distinct differences in other points were traced in the behaviour of the two classes of organisms. In this communication the study is extended to *Staphylococcus aureus* cultures with the object of obtaining general rules concerning the oxidation-reduction potential changes occurring in bacterial cultures. Staphylococci, like the organisms previously studied, may be cultivated under a variety of aerobic and anaerobic conditions, but most luxuriant growth is observed when free access to oxygen is provided. The very wide range of values of oxygen tension over which many micro-organisms can multiply contrasts with the relatively narrow limits within which higher organisms can survive. This ease of adaptation to different respiratory conditions confers on bacteria the property of great variation in behaviour.

METHODS.

The methods and apparatus employed have been precisely the same as those previously described [Hewitt, 1930, 1]. 8 cc. of medium were used in each experiment and the inoculum was 0·1 cc. of a 24-hour infusion broth culture of *Staph. aureus*.

RESULTS.

Peptone infusion broth (Fig. 1). In ordinary stock horse-flesh infusion broth containing 2 % of Difco peptone the electrode potential fell to the level, $E_h = -0{\cdot}14$ v., within 9 hours. After this initial drop, the potential fell slightly during the next 140 hours, showing no tendency to rise. This behaviour is similar to that of cultures of *C. diphtheriae* in which the potential remains at a low level for a long period, but quite different from that of haemolytic streptococci. In the latter case the potential begins to rise immediately after the cessation of active proliferation. A distinct point of inflection on the potential-time curves is seen (Fig. 2) indicating reduction processes occurring in two stages.

Freshly heated broth (Fig. 2). Infusion broth was heated for 30 minutes in a boiling water-bath and cooled immediately before use. The potential fell much more rapidly and to a lower level than in unheated broth. This phenomenon was observed with haemolytic streptococci also, but not with *C. diphtheriae*.

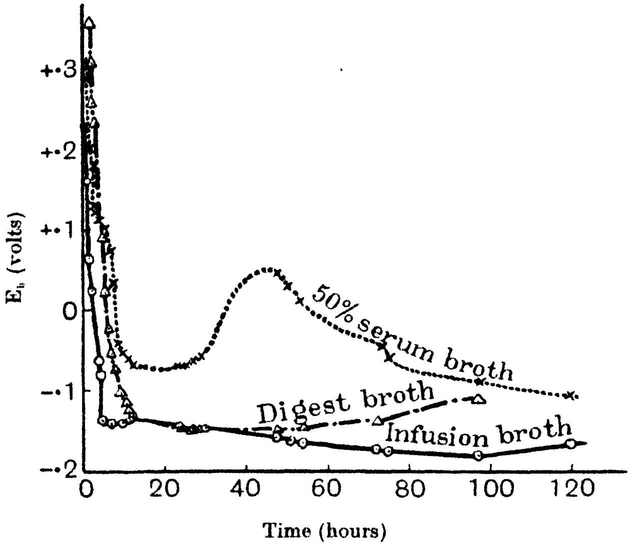

Fig. 1. Aerobic cultures of *Staph. aureus*.

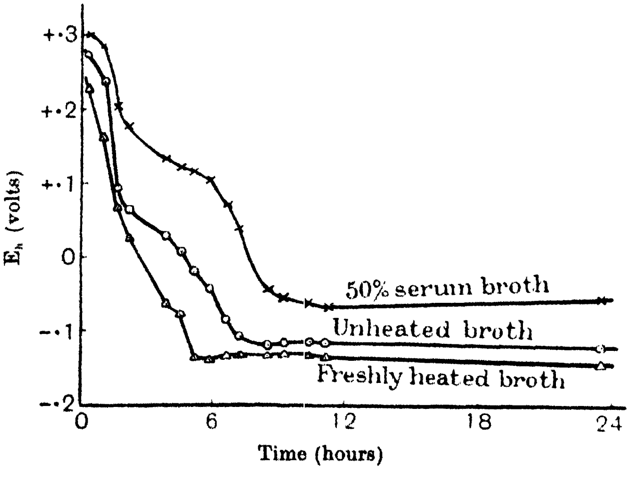

Fig. 2. Aerobic cultures of *Staph. aureus*.

Tryptic digest broth (Fig. 1). The potential fell quite rapidly in cultures in Douglas's tryptic digest medium. With haemolytic streptococci this sample of broth had a slight oxygen-carrying effect but it had very little with staphylococcal cultures.

Aerated cultures (Fig. 3). When cultures were aerated continuously and vigorously by means of Ridley's [1928] mixing apparatus growth was much more luxuriant than in stationary cultures. The potential fell rather more slowly and not to as low a level as in stationary aerobic cultures. The difference in potentials reached in aerated and aerobic cultures is much less than in the case of haemolytic streptococci but greater than with *C. diphtheriae.*

Table I. *Minimum electrode potentials reached in cultures.*

	E_h in volts		
	Streptococci	Staphylococci	*C diphtheriae*
Aerobic	− 0·16	− 0·155	− 0·20
Aerated	+ 0·12	− 0·055	− 0·15
Difference	0·28	0·10	0·05

The curve obtained with aerated cultures of haemolytic streptococci is given in Fig. 3 for comparison.

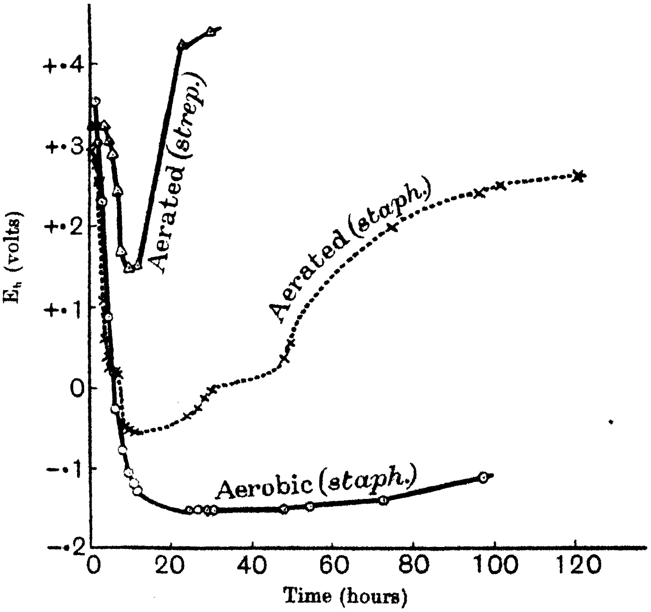

Fig. 3. Broth cultures of *Staph. aureus* and haemolytic streptococci.

Serum broth (Figs. 1 and 2). In 50 % serum broth cultures the potential did not fall to as low a level as in plain broth cultures. The form of the potential-time curves is similar to that obtained with haemolytic streptococci and possesses the same points of inflection. A further interesting observation is the second fall in potential in serum broth cultures after some 48 hours' growth. This may be due to breakdown of serum-proteins by proteolytic enzymes possessed by staphylococci.

Glucose broth (Fig. 4). The potential of aerobic cultures in 1 % glucose broth falls fairly rapidly but not to as low a level as in plain broth, and then rises, probably owing to the accumulation of acid in the culture. This phenomenon was observed also with haemolytic streptococci and *C. diphtheriae*.

In aerated glucose broth cultures the potential did not fall to as low a level as in the stationary glucose broth culture (minimum value $E_h = +0{\cdot}01$ v. in place of $-0{\cdot}08$ v.). After a period of increase in potential, however, probably associated with acid production, the potential fell for a second time and after 3–6 days' growth the potential in the aerated culture was actually lower than in the stationary aerobic culture, despite the oxidising effect of the increased oxygen supply in the aerated culture. It was found that the stationary culture had p_H 5·4, whilst in the aerated culture the p_H was 7·0 and growth was more luxuriant. It is evident, therefore, that the extra oxygen supply in the aerated culture had enabled the organisms to utilise the acids liberated during the glucose fermentation. The oxidation of lactate, etc., to carbonate accounts for the more alkaline reaction in the aerated culture.

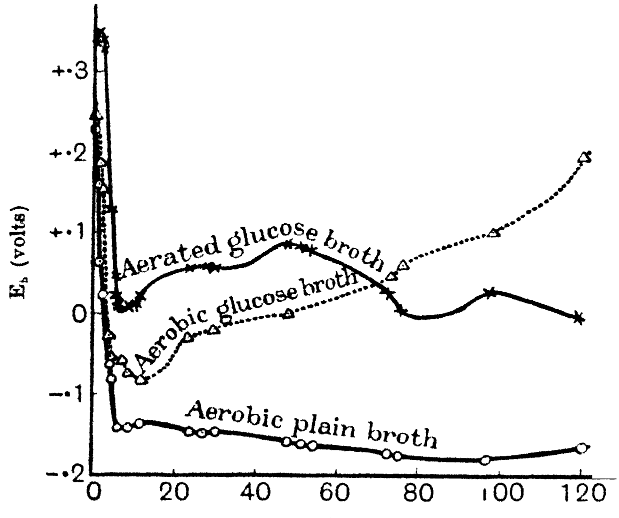

Fig. 4. Aerobic cultures of *Staph. aureus*.

Lactate-fumarate broth. In broth containing 1 % of sodium lactate and fumarate the potential fell to the same level as in plain broth but rose fairly rapidly. The reaction did not become alkaline and growth was relatively poor, indicating that the organisms were unable to utilise the acids in stationary aerobic cultures to any great extent.

Glycerol broth (Fig. 5). Good growth was observed in stationary aerobic 1 % glycerol broth cultures and the potential-time curve was practically indistinguishable from that of a plain broth culture. The culture did not become as acid as glucose broth cultures (p_H 6·6 in place of 5·4).

Thiodiglycollate broth (Fig. 5). In broth cultures containing 1 % of sodium thiodiglycollate the potential fell rather more slowly and did not reach the low level reached in plain broth cultures. There were also points of inflection on the curve similar to those obtained with haemolytic streptococci.

DISCUSSION.

Staphylococci multiply most readily under conditions of abundant oxygen supply, and are vigorous reducing agents. In this they resemble *C. diphtheriae*; both organisms are more easily able to maintain intense reducing conditions in the presence of a generous oxygen supply than are haemolytic streptococci. In addition, neither *C. diphtheriae* nor staphylococci form peroxide: therefore the potential-time curves of cultures of these organisms remain flat at a low level of potential after the logarithmic phase of growth, whereas with streptococci the curve immediately begins to rise.

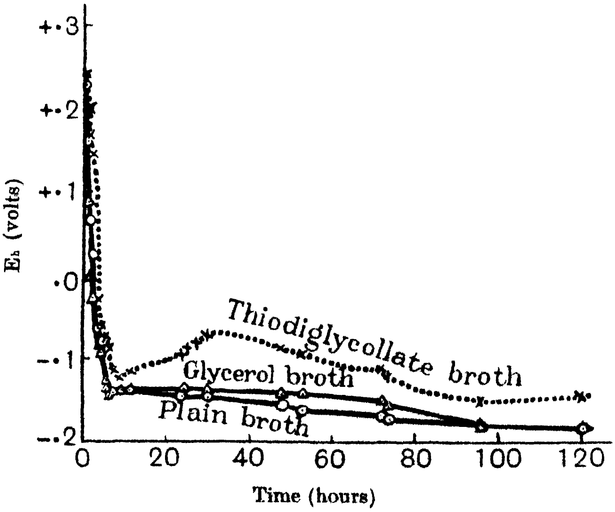

Fig. 5. Aerobic cultures of *Staph. aureus.*

Staphylococci resemble streptococci and differ from *C. diphtheriae* in the effect of freshly heated broth. In the case of the two former organisms the potential falls more rapidly and to a lower level in the freshly boiled broth. With *C. diphtheriae* no effect is seen.

With all three organisms the potential does not fall to as low a level in serum broth cultures as in plain broth. The oxygen-carrying effect of serum-proteins appears therefore to be a general one.

In glucose broth cultures also the three organisms are similar in that the potential does not fall to as low a level as in plain broth. With staphylococci, however, another effect is seen. Acids liberated during glucose fermentation are utilised much more readily in vigorously aerated cultures than in stationary aerobic cultures.

The presence of glycerol appears to have little effect on the potential-time curves of cultures, does not lead to acid production, and is therefore quite different in its effects from that of glucose.

When staphylococcal cultures are sealed autolysis proceeds and the culture clears. Little effect on the potential is observed, which suggests that the potential of the intracellular contents is very little different from that of the medium, under the conditions employed.

For comparison with previous results there are given in the following table the values of r_H (logarithm of reciprocal of partial pressure of hydrogen in equilibrium with the system) reached in *Staphylococcus aureus* cultures.

	Approximate r_H after					
Nature of culture	6 hrs.	12 hrs.	24 hrs.	48 hrs.	72 hrs.	120 hrs.
Unheated broth culture	12	10	10	10	10	9·5
Freshly heated culture	9·7	9·7	9·3	9	8·5	8·0
Aerated broth culture	15	12	12·5	15	20·5	22

SUMMARY.

1. *Staphylococcus aureus* resembles *C. diphtheriae* in maintaining more intense reducing conditions when the oxygen supply is abundant than haemolytic streptococci.

2. Probably owing to lack of peroxide-formation the potential in staphylococcal cultures remains at a low level for a long period, again like diphtheria and unlike streptococci.

3. In freshly heated broth the potential falls more rapidly in staphylococcal cultures, as with streptococci but not with *C. diphtheriae*.

4. Serum has an oxygen-carrying effect in cultures of staphylococci as with the other organisms investigated.

5. It is found with staphylococci, as with streptococci and *C. diphtheriae*, that the potential does not fall to as low a level in glucose broth as in plain broth; but with staphylococci an abundant oxygen supply facilitates the utilisation of acids liberated during glucose fermentation.

6. Glycerol, unlike glucose, has little effect on the potential-time curve.

The author wishes to acknowledge his indebtedness to Dr R. G. White and Dr E. W. Todd for their ready help, interest and encouragement.

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LXXVIII. THE FAT-SOLUBLE VITAMINS OF MILK.

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Report to the Medical Research Council.

(*Received May 6th, 1930.*)

Some years ago McCollum, Simmonds and Steenbock [1917] made the observation that fat-free milk, when included in a diet consisting of purified food substances, promoted growth and prevented decline of animals and, further, that melted butter-fat lost its growth-promoting capacity after being thoroughly agitated with twenty successive portions of water. This led them to suspect that the "fat-soluble A factor" might be appreciably soluble in water. Later, Steenbock, Boutwell and Kent [1918] were able to demonstrate that the "fat-soluble vitamin" was not actually removed from the butter by the water but that it was destroyed in the process of washing. Drummond, Coward and Watson [1921] using a basal diet free from both fat-soluble vitamins (the separate identity of the fat-soluble vitamins was not appreciated at that time) came to the conclusion that butter was less potent as a source of "fat-soluble A factor" than the same amount of fat supplied in the original milk and that the loss incurred was partly mechanical and partly due to destruction. As far as we are aware no strictly quantitative experiments have since been carried out to demonstrate whether the growth with the "fat-free" milk observed by McCollum, Simmonds and Steenbock was due to the presence of the fat-soluble vitamins present in the residual fat of the skim milk, which we presume their milk contained, or whether one or both of the fat-soluble vitamins of milk-fat is really appreciably soluble in water. The nature of an enquiry in which one of us (S.S.Z.) is at present engaged necessitated that this should be satisfactorily established and the experiments described in this communication were undertaken with this end in view.

METHODS.

The morning milk from a typical herd of nine short-horn cows, which are kept for the study of normal milk at the National Institute for Research in Dairying, Shinfield, Berks., was used in this experiment. The sample was collected on May 13th, 1929. The cows were on pasture day and night and in addition received a mixture consisting of dried grains two parts and yellow maize germ meal one part.

This concentrate mixture was given to six cows which were in full milk, in quantities which averaged 6·5 lb. per day per cow, varying from 0 to 12 lb. according to the milk yield.

The milk contained 3 % fat and 12·05 % total solids. 25·75 lb. of the whole milk was dried in a vacuum pan under diminished pressure (75·75 cm.) for 5 hours; the temperature of the milk never rose above 36°. When the milk had reached a suitable concentration, the drying was continued in an evacuated desiccator over P_2O_5 at 37° until the water content was 22·15 %. It was then transported to the Lister Institute, London, and kept there in the cold room during the entire testing period. Likewise the separated milk from 36·1 lb. of the original milk, containing 0·1 % of fat was concentrated to a water content of 32·75 %. The separated cream was churned and the butter-milk thus obtained concentrated. The latter concentrate was added in equivalent quantities to the dried separated milk during the tests. The butter contained 11·7 % water. The dried milks were acid and had an odour of butyric acid.

In the butter, vitamin A was determined as previously described [Golding, Soames and Zilva, 1926] by the modified Steenbock method, daily doses of 0·1 g., 0·2 g. and 0·6 g. being employed. The estimation of vitamin A in the whole and in the skim milk plus butter-milk was carried out by administering daily the dose of dried milk mixed with an equal quantity of the basal diet [see Golding, Soames and Zilva, 1926] from which the caseinogen had been eliminated. After the animals had consumed the dose, the basal diet was offered *ad lib.* In the case of the whole milk, doses approximately equivalent to 0·05 g., 0·1 g. and 0·2 g. and in that of the skim milk plus butter-milk to 0·2 g., 0·5 g. and 0·7 g. of butter were tested. The animals apart from their doses consumed their basal diet willingly and in ample quantities (12–15 g. *per diem*).

Vitamin D in the whole and in the skim milk could not be assayed by a method in which McCollum's diet No. 3143 was used, because the daily consumption of the necessary quantities of dried milk equivalent to the butter doses would have also entailed the consumption of significant quantities of phosphorus, in which this diet is characteristically deficient. In fact, preliminary tests have actually shown that no satisfactory results could be obtained under the circumstances. Recourse was had to another method, namely that of Soames and Leigh-Clare [1928]. This method is based in the main on the increase in weight in young rats produced by the addition of vitamin D to a diet free from this vitamin but otherwise adequate. The basal diet employed in this research was Soames and Leigh-Clare's diet No. 2 and was made up as follows:

	Parts by weight
Wheat starch	43·7
Inactivated caseinogen ...	14·3
Cotton seed oil (hardened)	12·4
Yeast	8·0
Salt mixture	5·0
Lemon juice	5·0
Wheat germ	20·0
Water	50·0

The daily doses of dried milk were given in equivalent quantities of the following mixture:

	Parts by weight
Starch...	79
Hardened cotton seed oil ...	21
Yeast	14
Salt mixture	9
Lemon juice	9
Wheat germ	36
The dried whole or skim milk	125
Water...	82

After the consumption of the test dose, the above basal diet was offered *ad lib.* and quantities adequate to induce good growth in presence of sufficient vitamin D were consumed by the animals. In addition to the growth index, the ratio (A/R ratio) of the amount of mineral ash to the amount of organic material contained in the fat-extracted bone [see Chick, Korenchevsky and Roscoe, 1926] was also determined as suggested by Soames and Leigh-Clare.

DISCUSSION OF RESULTS.

Vitamin A.

The skim milk to which, as already mentioned, the butter-milk from the churning of the butter was added, contained of course a certain amount of the milk-fat. Thus the highest dose of 2·8 g. which was equivalent to 0·68 g. of butter contained 0·066 g. of fat, the second dose of 2·1 g. which was equivalent to 0·51 g. of butter contained 0·050 g. of fat, and the lowest dose of 0·7 g. which was equivalent to 0·17 g. of butter contained 0·016 g. of fat. Were any appreciable quantity of vitamin A associated in the milk after separation or in the butter-milk after churning with constituents other than fat, or should the vitamin have been more concentrated in the residual fat than in the butter, it would have been reflected in the growth curves of the rats receiving the "skim milk." By examining the growth curves of these animals, however (Fig. 1), it is seen that this was not the case. but that the growth obtained was more or less proportional to the residual fat present in the doses. Thus the lowest dose containing 0·016 g. of residual fat induced no appreciable growth, but the higher doses gave much better growth. This is particularly marked in the case of the highest dose containing 0·066 g. of residual fat. In fact the growth obtained with this dose was almost as good as that obtained with 0·1 g. of the butter (group VI, Fig. 2), which suggests that the minimum dose of milk-fat lies between these quantities. The weight curves of the rats receiving the whole milk (Fig. 2) also indicate that the vitamin A content of the doses was proportional to their milk-fat content, since the daily minimum amount of milk-fat necessary to induce optimum growth lies in this case between 0·05 g. (group III, Fig. 2) and 0·10 g. (group II, Fig. 2), a minimum also indicated by the growth curves obtained with the "skim milk" and with the butter.

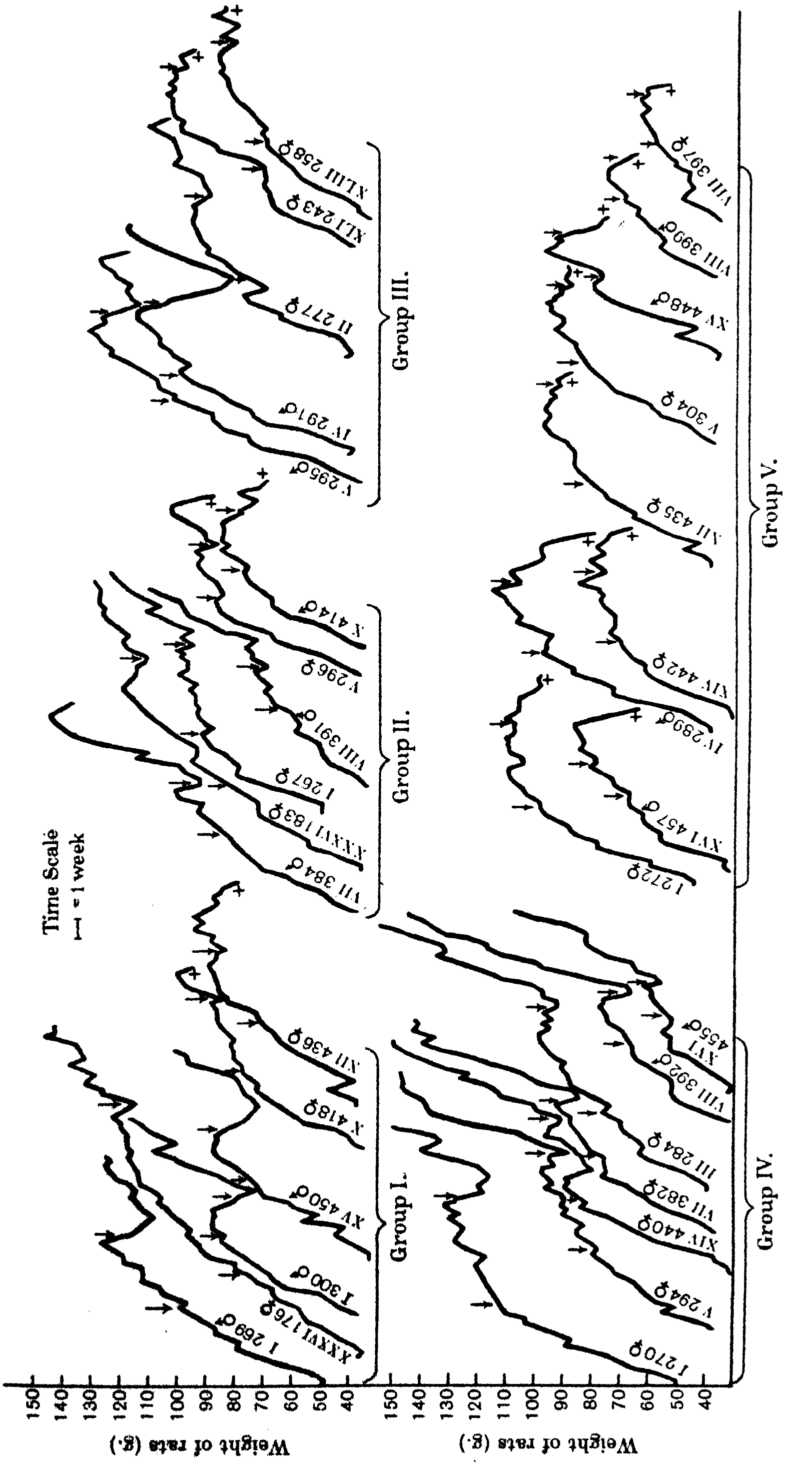

Fig. 1.

Group. I. Daily dose 2·80 g. of dried skim milk and butter-milk, equivalent to 0·68 g. of butter and containing 0·066 g. of milk-fat.
Group II. Daily dose 2·10 g. of dried skim milk and butter-milk, equivalent to 0·51 g. of butter and containing 0·050 g. of milk-fat.
Group III. Daily dose 0·70 g. of dried skim milk and butter-milk, equivalent to 0·17 g. of butter and containing 0·016 g. of milk-fat.
Group IV. Positive controls; 5 drops of cod-liver oil daily. Group V. Negative controls.

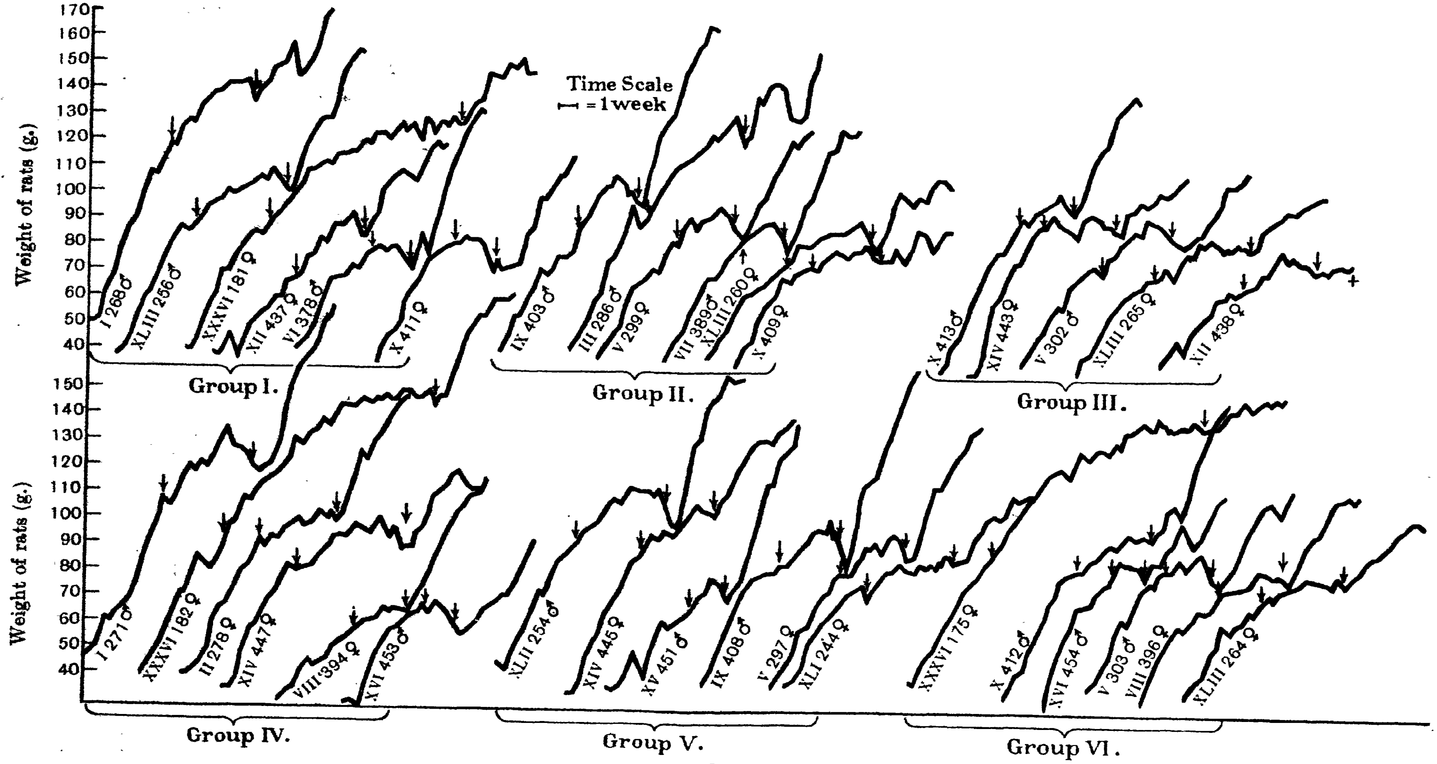

Fig. 2.

Group I. Daily dose 1·0 g. of dried whole milk, equivalent to 0·20 g. of butter and containing 0·21 g. of milk-fat.
Group II. Daily dose 0·50 g. of dried whole milk, equivalent to 0·10 g. of butter and containing 0·10 g. of milk-fat.
Group III. Daily dose 0·25 g. of dried whole milk, equivalent to 0·05 g. of butter and containing 0·05 g. of milk-fat.
Group IV. Daily dose 0·6 g. of butter. Group V. Daily dose 0·2 g. of butter. Group VI. Daily dose 0·1 g .of butter.
First arrow indicates beginning of the administration of irradiated cotton seed oil (vitamin D), second arrow that of the test dose.
+ denotes death.

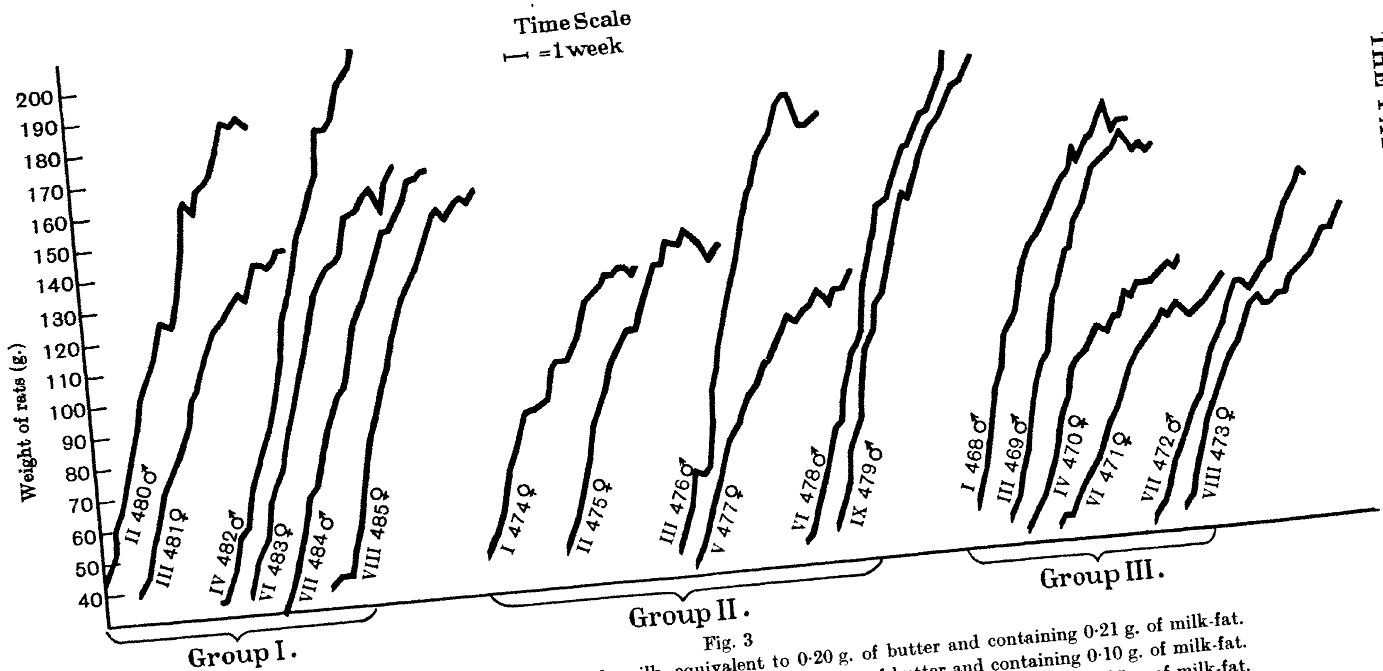

Fig. 3

Group I. Daily dose 1·0 g. of dried whole milk, equivalent to 0·20 g. of butter and containing 0·21 g. of milk-fat.
Group II. Daily dose 0·50 g. of dried whole milk, equivalent to 0·10 g. of butter and containing 0·10 g. of milk-fat.
Group III. Daily dose 0·25 g. of dried whole milk, equivalent to 0·05 g. of butter and containing 0·05 g. of milk-fat.

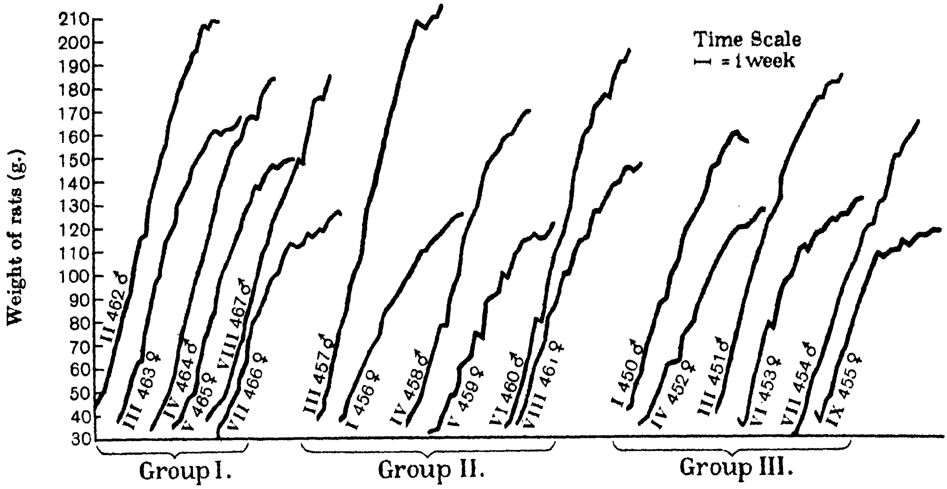

Fig. 4.

Group I. Daily dose 0·6 g. of butter.
Group II. Daily dose 0·2 g. of butter.
Group III. Daily dose 0·1 g. of butter.

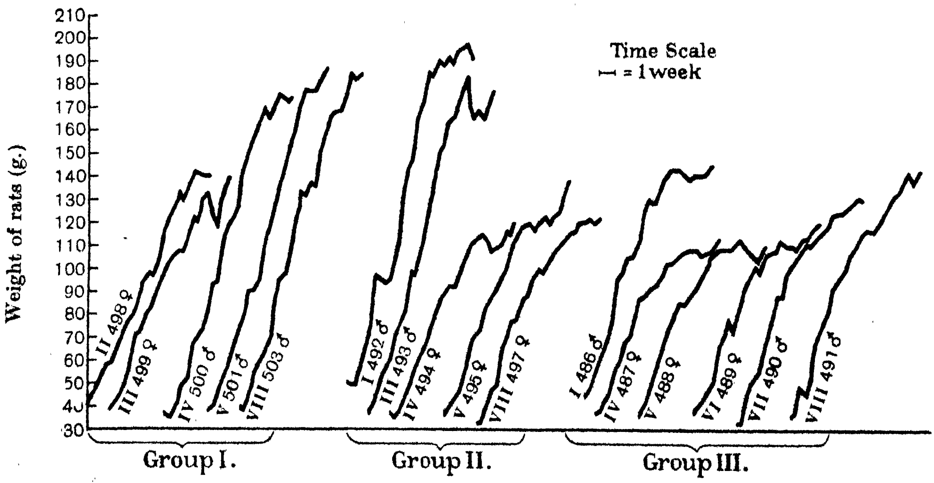

Fig. 5.

Group I. Daily dose 2·80 g. of dried skim milk and butter-milk, equivalent to 0·68 g. of butter and containing 0·066 g. of milk-fat.

Group II. Daily dose 2·10 g. of dried skim milk and butter-milk, equivalent to 0·51 g. of butter and containing 0·050 g. of milk-fat.

Group III. Daily dose 0·70 g. of dried skim milk and butter-milk, equivalent to 0·17 g. of butter and containing 0·016 g. of milk-fat.

Vitamin D.

Although the limitations of the method for the determination of vitamin D employed in this research have not yet been fully studied, we are of the opinion that the results obtained leave little doubt that, as in the case of vitamin A, vitamin D is associated entirely with the milk-fat. The growth-promoting capacity of the skim milk was again proportional to the amount of residual fat present in the dose. In these tests the minimum daily dose which induced optimum growth (as compared with the positive control group) was between 0·05 g. and 0·066 g. (groups II and I, Fig. 5), which was also more or less the minimum dose indicated by the whole milk and butter tests (Figs. 3, 4 and 6). The analysis of bones of these rats (Table I and Fig. 7) showed that the *A*/*R* ratios were also more or less proportional to the amount of milk-fat present in the dose. These experiments showed that an optimum average *A*/*R* ratio was attained only when the rats received a daily dose of 0·2–0·6 g. of butter, a dose higher than that required to induce optimum growth. The animals on a dose of dried "skim milk" containing 0·066 g. of milk-fat (group IX, Fig. 7) showed a somewhat higher average *A*/*R* ratio than the animals receiving 0·1 g. of butter equivalent to 0·088 g. of fat (group I, Fig. 7)[1]. This, however, we consider as an error inherent to the method rather than an indication that the residual fat in the skim milk was more potent than the butter-fat.

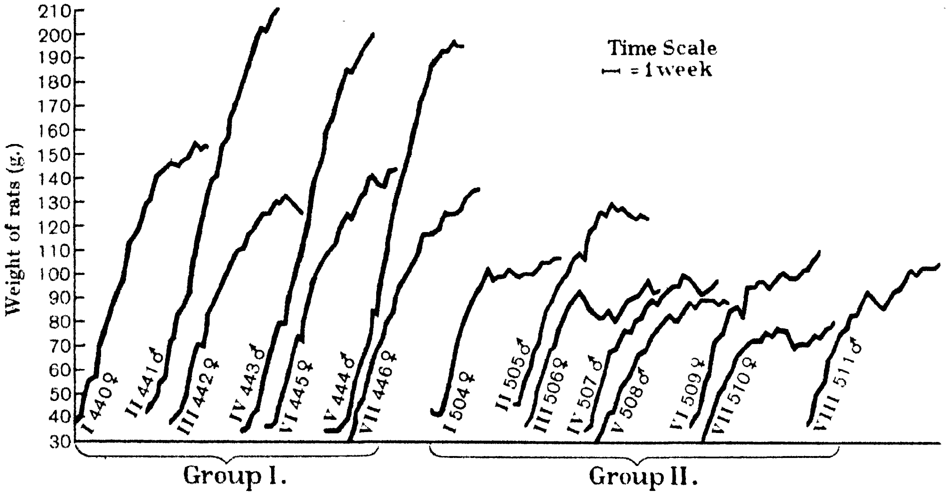

Fig. 6.

Group I. Positive controls; 5 drops of cod-liver oil daily.
Group II. Negative controls.

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[1] Also the animals on the dose 2·1 g. of dried skim milk containing 0·05 g. of milk-fat showed a slightly higher ratio than the animals receiving the dose 0·25 g. of whole dried milk containing 0·05 g. of fat.

Table I.

Negative controls	
Rat	*A/R*
I 504 F.	1·18
II 505 M.	1·18
III 506 F.	1·20
IV 507 M.	1·06
V 508 M.	1·04
VI 509 F.	1·36
VII 510 F.	1·23
VIII 511 M.	1·17
Average	**1·18**

Positive controls		
Dose	Rat	*A/R* Ratio
5 drops cod-liver oil daily	I 440 F.	1·59
	II 441 M.	1·45
	III 442 F.	1·55
	IV 443 M.	1·58
	V 444 M.	1·42
	VI 445 F.	1·59
	VII 446 F.	1·59
	VIII 447 M.	—
	Average	**1·54**

Butter			Dried whole milk			Dried skim and butter-milk		
Dose	Rat	*A/R*	Dose	Rat	*A/R*	Dose	Rat	*A/R*
0·1 g.	I 450 M.	1·32	0·25 g. approx. equiv. to 0·05 g. butter and contained 0·05 g. of milk-fat	I 468 M.	1·28	0·7 g. approx. equiv. to 0·17 g. butter and contained 0·016 g. of milk-fat	I 486 M.	1·17
	III 451 M.	1·33		III 469 M.	1·28		IV 487 F.	1·34
	IV 452 F.	1·54		IV 470 F.	1·37		V 488 F.	1·26
	VI 453 F.	1·48		VI 471 F.	1·36		VI 489 F.	1·26
	VII 454 M.	1·39		VII 472 M.	1·37		VII 490 M.	1·41
	IX 455 F.	1·37		VIII 473 F.	1·40		VIII 491 M.	1·29
	Average	**1·40**		Average	**1·34**		Average	**1·29**
0·2 g.	I 456 F.	1·36	0·5 g. approx. equiv. to 0·1 g. butter and contained 0·1 g. of milk-fat	I 474 F.	1·33	2·1 g. approx. equiv. to 0·51 g. butter and contained 0·05 g. of milk-fat	I 492 M.	1·37
	III 457 M.	1·42		II 475 F.	1·44		III 493 M.	1·40
	IV 458 M.	1·48		III 476 M.	1·40		IV 494 F.	1·43
	V 459 F.	1·46		V 477 F.	1·37		V 495 F.	1·47
	VI 460 M.	1·41		VI 478 M.	1·36		496	—
	VIII 461 F.	1·55		IX 479 M.	1·38		VIII 497 F.	1·61
	Average	**1·45**		Average	**1·38**		Average	**1·46**
0·6 g.	II 462 M.	1·42	1·0 g. approx. equiv. to 0·2 g. butter and contained 0·21 g. of milk-fat	II 480 M.	1·42	2·8 g. approx. equiv. to 0·68 g. butter and contained 0·066 g. of milk-fat	II 498 F.	1·46
	III 463 F.	1·65		III 481 F.	1·46		III 499 F.	1·56
	IV 464 M.	1·47		IV 482 M.	1·42		IV 500 M.	1·41
	V 465 F.	1·06		VI 483 F.	1·61		V 501 M.	1·45
	VII 466 F.	2·36		VII 484 M.	1·40		502	—
	VIII 467 M.	1·40		VIII 485 F.	1·59		VIII 503 M.	1·53
	Average	**1·56**		Average	**1·48**		Average	**1·48**

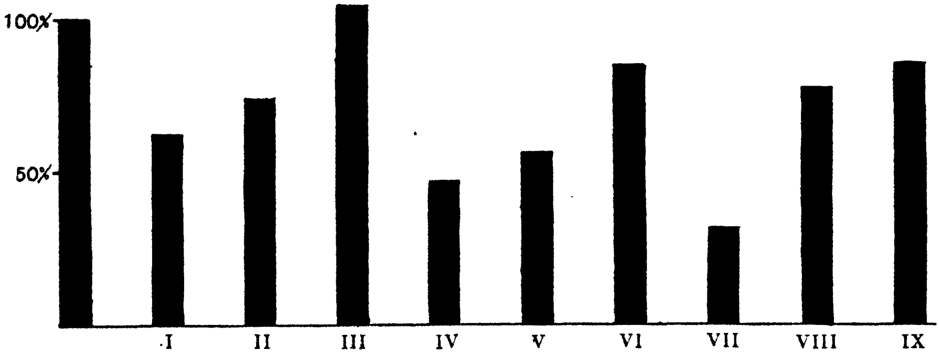

Fig. 7.

Percentage differences between average ratio of groups of animals and negative controls, ash/organic residue, not fat. (Difference between average ratio of positive group receiving daily 5 drops of cod-liver oil and negative group of each series = 100 %.)

Group I. Daily dose 0·1 g. of butter.

Group II. Daily dose 0·2 g. of butter.

Group III. Daily dose 0·6 g. of butter.

Group IV. Daily dose 0·25 g. of dried whole milk, equivalent to 0·05 g. of butter and containing 0·05 g. of milk-fat.

Group V. Daily dose 0·50 g. of dried whole milk, equivalent to 0·10 g. of butter and containing 0·10 g. of milk-fat.

Group VI. Daily dose 1·0 g. of dried whole milk, equivalent to 0·20 g. of butter and containing 0·21 g. of milk-fat.

Group VII. Daily dose 0·70 g. of dried skim milk and butter-milk, equivalent to 0·17 g. of butter and containing 0·016 g. of milk-fat.

Group VIII. Daily dose 2·10 g. of dried skim milk and butter-milk, equivalent to 0·51 g. of butter and containing 0·050 g. of milk-fat.

Group IX. Daily dose 2·80 g. of dried skim milk and butter-milk, equivalent to 0·68 g. of butter and containing 0·066 g. of milk-fat.

SUMMARY.

The results obtained in these experiments suggest definitely that the entire vitamin A and vitamin D content of milk is associated with the milk-fat. There are no indications that these vitamins are more concentrated in one fraction of the milk-fat than in another, nor that there is a significant loss in either vitamin due to separating or churning, and consequently by testing the butters it is possible to obtain the approximate relative potency of milks. These experiments further suggest the utility of the Soames and Leigh-Clare method for the determination of vitamin D. The limitations of this method are being studied further.

We are indebted to Miss Dearden for the churning of the milk.

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LXXIX. VITAMIN A AND CAROTENE.

V. THE ABSENCE OF THE LIVER OIL VITAMIN A FROM CAROTENE.

VI. THE CONVERSION OF CAROTENE TO VITAMIN A *IN VIVO*.

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From the Nutritional Laboratory, Cambridge.

Report to the Medical Research Council.

(*Received May 3rd, 1930.*)

V. The absence of the liver oil vitamin A from carotene.

There is now general agreement that carotene of the highest purity so far attained possesses intense vitamin A activity [Euler, Euler and Hellström, 1928; Moore, 1929, 1, 2; Collison, Hume, Smedley-MacLean and Smith, 1929; Kawakami and Kimm, 1929; Hume and Smedley-MacLean, 1930], and the negative results of early workers must be attributed either to failure to supply vitamin D in the test diet, or more probably to insufficient precautions against oxidation of the pigment during the administration of the test doses.

Nevertheless it has been obvious for many years that carotene cannot be identified with the vitamin A of liver oils, since this factor must presumably be an almost colourless substance, differing notably from carotene in its solubility and adsorption properties. Thus vitamin A, present in unknown amounts in the pale orange-coloured liquid concentrates of cod-liver oils, is readily soluble in almost all organic solvents, including methyl alcohol and natural fats. Carotene, on the other hand, although not greatly exceeding the best cod-liver oil concentrates in physiological activity, is so deeply pigmented as to appear almost black in the pure state, and is readily soluble only in such solvents as chloroform, carbon disulphide and hot cyclohexane, being sparingly soluble in natural fats and almost insoluble in methyl alcohol. Similarly carotene is much more readily absorbed from its solutions by charcoal than vitamin A, the difference being so great as to afford an easy means of separation [Stephenson, 1920]. It is necessary, therefore, to account for the possession of a common physiological activity by two very divergent sources, and in the first place to ensure that the activity of carotene is not due to the presence of the liver oil factor in an adsorbed state, or in some other condition that would alter its normal solubility properties.

In supporting the contention that the activity of carotene samples might be due to contamination with the familiar form of vitamin A, Dulière, Morton and Drummond [1929, 1] described a careful colorimetric and spectroscopic differentiation of carotene from the vitamin A of cod-liver oil. They found, in confirmation of Euler, Euler and Hellström, that the blue colours produced in the $SbCl_3$ reaction were of slightly different shade, that of carotene being characterised by an absorption band at 590 $\mu\mu$, that of vitamin A by a band at 608–612 $\mu\mu$, while in regard to the ultra-violet absorption spectra vitamin A was associated with a band at 320–330 $\mu\mu$, which was absent in the case of carotene. Carotene and the vitamin were therefore certainly different, but since it was also found that less pure specimens of carotene, similar to those found active by other workers, differed but little in their spectroscopic behaviour from highly purified samples, the additional suggestion was made that the presence of effective amounts of vitamin A in carotene might escape spectroscopic detection by reason of more intense superimposed absorption due to the pigment itself [Dulière, Morton and Drummond, 1929, 2].

Although Hume and Smedley-MacLean [1930] have now shown that even the most carefully purified carotene exhibits vitamin A activity, provided that the test doses are made up in a solvent in which decomposition does not take place, the above criticism would still seem to apply, since the persistence of traces of the liver oil vitamin A in carotene of the highest melting point might well be concealed if the absorptions due to the pigment were in fact of relatively overwhelming density. Capper [1930] has already shown that this condition does not hold good in regard to the absorption band at 328 $\mu\mu$. In the experiments described below it will similarly be shown that the blue colour given by carotene in the $SbCl_3$ reaction is of insufficient relative density to conceal the presence of effective amounts of the vitamin. This conclusion is in agreement with the previous qualitative observation that no sign of a maximum at 610–630 $\mu\mu$ could be observed in the colours produced in the $SbCl_3$ reaction by highly active samples of carotene [Moore, 1929, 1].

EXPERIMENTAL.

Method. Specimens of carotene (M.P. 174° and 178°) were compared colorimetrically with specimens of cod-liver oil concentrates (kindly supplied by Messrs Joseph Nathan and Co., Ltd., and Messrs Lever Brothers, Ltd.) using the technique previously described [Moore, 1929, 2]. The same materials were then tested biologically at doses graded to run parallel to the colorimetric values.

Colorimetric results. The chromogenic activities of the various materials were determined several times in each case and may be approximately represented by the following values[1].

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[1] Although the tintometer technique certainly yields results of great value to the individual worker some uncertainty must be felt in expressing the values so obtained on an absolute scale. Apart from avoidable complications arising from variations in temperature and reagent [Wokes and Willimott, 1927; Evers, 1929, 1, 2] or from accelerated fading due to the presence of unsaturated acids [Norris and Danielson, 1929; Norris and Church, 1930] a personal element cannot be excluded in matching the shades produced. The results presented in the test, however, were obtained with the utmost care on the same occasion, and can in any case be regarded as giving satisfactory relative values.

	Blue units per mg. per "1 cm. cube" $SbCl_3$ reagent
Carotene (M.P. 174°)	180 at 590$\mu\mu$
„ (M.P. 178°)	180 „
Concentrate A	180 at 610–630$\mu\mu$
„ B	270 „ „

Biological tests. The biological work was carried out in several separate sections, rats nos. 1, 2, 3, 4 (albinos), nos. 5, 6, 7, 8, 9, 10, 11, 12 (albinos), nos. 13, 14, 15 (piebalds), and nos. 16 (piebald), 17, 18, 19 (albinos), being used in successive series of experiments. The usual curative technique was invariably employed, all the animals receiving the vitamin A-free diet previously employed, with the exception of rats nos. 13, 14, 15, which were kept under the care of Miss V. R. Leader at the Sir William Dunn Institute, where they received a slightly different basal diet (caseinogen 23 %, rice starch 40 %, cane sugar 17 %, palm kernel oil 15 %, salt mixture 5 %, + marmite extract 7·5 %).

Since all the materials had been found in earlier experiments to give positive results at 0·01 mg. per rat daily, attention was directed to dosages slightly below this level. The test doses were made up in arachis oil, one of the carotene samples (M.P. 174°) being dissolved by warming in the oil, the other (M.P. 178°) being added to the oil in hot cyclohexane, which was then removed under diminished pressure. During the experiment all the test solutions were kept in cold storage except when actually in use, and to ensure that no serious deterioration had taken place were tested by the $SbCl_3$ reagent at the conclusion of the experiment. The growth curves obtained are shown in Fig. 1.

DISCUSSION.

In the above experiments carotene was found to be effective at levels down to 0·004 mg. per rat per day, while, with the exception of a single rat recovering on 0·002 mg., lower doses were found to be ineffective. The more active of the two concentrates tested was effective in curing a single rat at 0·0033 mg. but negative results were obtained in all other cases. It would thus appear that weight for weight the carotene samples were of slightly greater biological activity than the concentrate.

From the parallel colorimetric determinations 0·004 mg. of carotene may be considered equivalent to 0·7 Lovibond blue unit at 590$\mu\mu$, while 0·0033 mg. of concentrate A corresponds to 0·9 blue unit at 610–630$\mu\mu$. The colour value given by a minimal dose of the pigment therefore appears to be slightly smaller than that given by a minimal dose of concentrate. Since the possibility of the concealment of effective amounts of the liver oil vitamin A in carotene would imply that the colour value given by a minimal dose of carotene should be much greater than that given by a minimal dose of the concentrate this result affords strong evidence against this view. When in addition the work of Capper on the absence of the 328$\mu\mu$ absorption band from carotene is taken into account the only reasonable conclusion to be reached is that the activity of the pigment certainly cannot be attributed to direct contamination with vitamin A, or at least with the chromogen associated with that factor in liver oils.

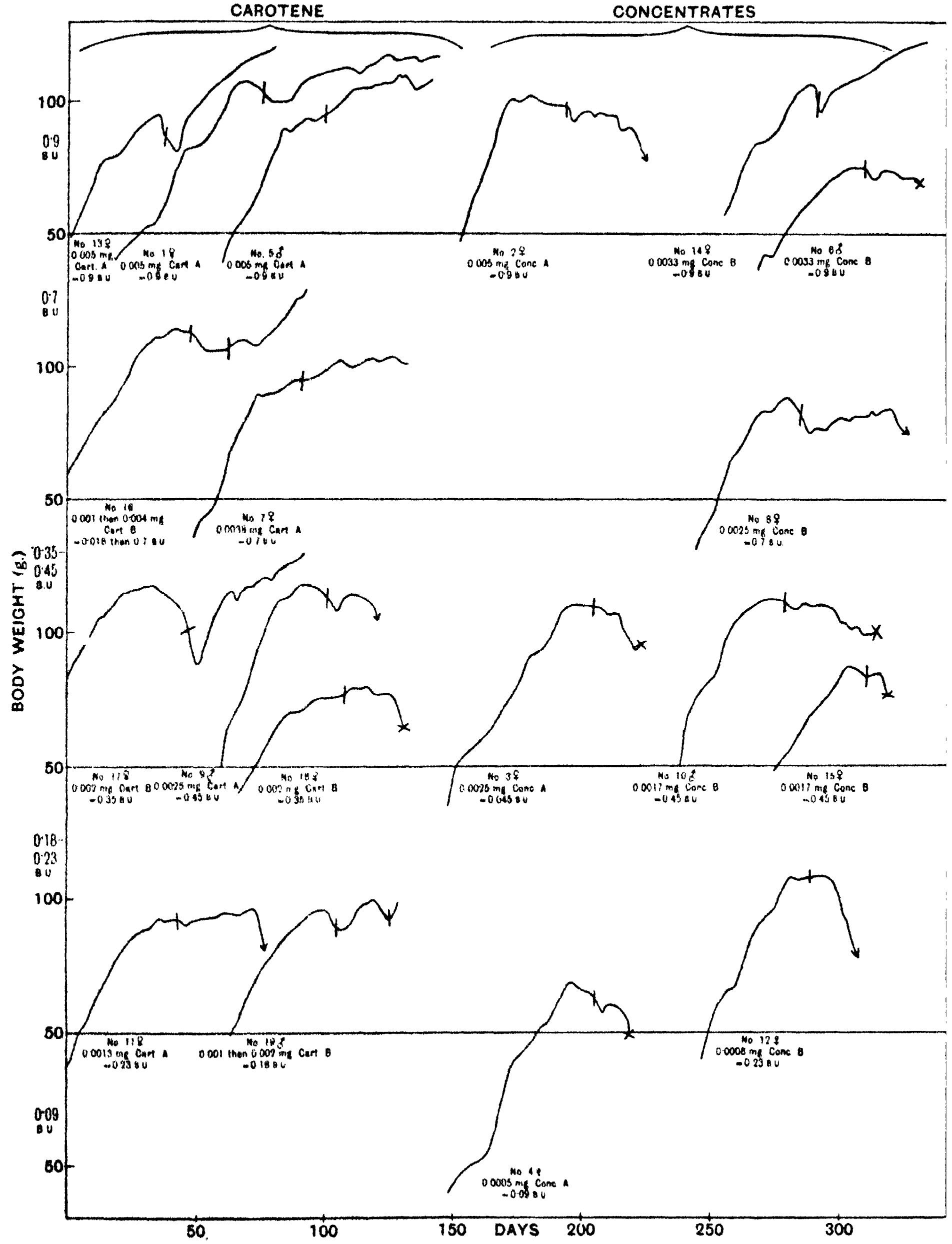

Fig. 1. The relative vitamin A potencies of carotene and cod-liver oil concentrates. B.U. = Lovibond blue ("1 cm. cube") unit. Cart. A = carotene (M.P. 174°). Cart. B = carotene (M.P. 173°). Conc. A, B = concentrate A, B.

VI. The conversion of carotene to vitamin A *in vivo*.

The question was once raised by Palmer [1919] as to whether it was logical to test the physiological activity of carotene in an animal, such as the rat, from which it was invariably absent. Answering the question in the negative Palmer and Kennedy [1920] attacked the question of the alleged identity of carotene with vitamin A by indirect means, and succeeded in proving conclusively that rats could thrive on diets almost completely freed from carotenoids. The assertion was therefore made that the pigment and vitamin were "neither identical nor necessarily associated."

The work of Euler, Euler and Hellström on the activity of carotene therefore seemed in conflict with preconceived ideas of the nature of the vitamin, while the later statement [Euler, Euler and Karrer, 1929, 1, 2] that excess of carotene could be stored in considerable amount in the liver oil of the rat was in direct contradiction to the finding of Palmer and Kennedy[1]. The Swedish workers, however, realised that carotene and the vitamin A of cod-liver oil were not identical, and suggested that several substances of carotenoid or "polyene" type might possess vitamin A activity.

In the first paper of this series [Moore, 1929, 1] it was pointed out that the "polyene" theory would imply an unexpectedly low order of specificity in vitamin A action, and the alternative suggestion was made that a chemical relationship between carotene and the vitamin might exist. Preliminary experiments supporting this view have already been described elsewhere [Moore, 1929, 3]. In the present paper more extensive evidence on the same point is presented.

EXPERIMENTAL METHOD.

The method adopted in proving the conversion of carotene to the vitamin was essentially as follows. Young albino rats were "run out" upon a basal diet deficient in vitamin A [Moore, 1929, 1] until the "plateau" condition indicative of exhaustion of reserves of the vitamin was attained. Certain of the rats were then killed in this condition. To the remaining rats, varying amounts of carotene, either in the purified state or as red palm oil or carrots, were administered until complete cures and considerable increases in body weight had been effected. These rats were then killed.

The livers of all the rats were dissected out, finely minced, and mixed with twice their bulk of 5 % aqueous KOH, after which they were placed in cold storage until a suitable opportunity for examination arose. Even proonged storage did not lead to deterioration, although in most cases examinations were carried out in the course of a few days. On removal from cold storage the gelatinous masses were gently warmed until almost complete solution of the liver tissues was effected. The solutions were then extracted four times with about twice their volume of ether. The combined extracts in each case were washed thoroughly three times with water, and if necessary dried by shaking with anhydrous Na_2SO_4. The ether was then removed by evaporation under diminished pressure in a current of nitrogen, and the residual liver oils tested immediately for carotene and vitamin A by colorimetric methods.

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[1] (*Note added in proof.*) In a recent communication accessible to the present author only after the above paper went to press, Euler and Rydbom [*Svensk. Kemisk. Tidskrift*, 1929, **41**, 223] have shown that when rabbits are fed carrots or carrot extracts a "pale yellow coloured carotenoid," and not carotene, is stored in the liver. Although no attempt has been made to identify this "carotenoid" with the classical vitamin A, it is obvious that this result is in complete agreement with the experimental findings described in the present paper in the case of the rat.

The colorimetric differentiation of carotene from vitamin A.

It should be noted that, since carotene and the liver oil vitamin A both give positive results when tested biologically, it is unavoidable that resource to colorimetric data should be made in studying the conversion of one to the other. The validity of the colour reaction of the vitamin has indeed been questioned from time to time by several workers, but Drummond and his colleagues [see Ahmad and Drummond, 1930] have generally shown that such criticisms have been quite unjustified.

Suppose we compare the natural yellow colour of carotene itself in chloroform solution with the blue colours which it produces when it is dissolved in the same volume of $SbCl_3$ reagent. A yellow to blue ratio of about 11 to 1 is then found. (The yellow colour of the sample tested is replaced on treatment with the $SbCl_3$ reagent by a much less intense blue colour.) On the other hand, when we compare the faintly yellow colours of cod-liver oil concentrates with the corresponding $SbCl_3$ blue colours a ratio yellow to blue of about 1 to 100 is found. (The concentrate when so dilute as to be almost colourless will produce on treatment with the $SbCl_3$ reagent an intense blue colour.) Thus when one constituent is present in a given material in predominant amount the yellow to blue ratio suggests at once whether this constituent is a carotenoid or the almost colourless vitamin, while in the case of equal mixtures a rough working approximation as to the probable proportion is afforded.

In the present work the yellow to blue ratio was used as a first indication of the nature of the chromogen present in the liver oils. Results were then confirmed by the position of the absorption band produced in the $SbCl_3$ reaction (carotene 590 $\mu\mu$; vitamin A 610–630 $\mu\mu$). Certain samples were then despatched to Mr N. S. Capper for examination in regard to the ultra-violet absorption, while others were employed in biological tests.

RESULTS.

The results obtained are shown in Table I. It will be seen that the liver oils of rats nos. 1 to 10, which had either died or been killed in a condition of acute avitaminosis A, invariably gave negative results when tested with the $SbCl_3$ reagent. The liver oils of rats nos. 11 to 14, in which growth had been restored by small doses of carotene, similarly gave negative results, as indeed would be expected at levels sufficient to meet immediate needs but insufficiently liberal to permit storage of the vitamin. On the other hand, in the case of the liver oils of rats nos. 15 to 22, which received either purified carotene, red palm oil, or fresh carrots in considerable excess of the minimal dosage, strongly positive reactions were given, which in all cases could be attributed to the appearance of large amounts of vitamin A, and not to the storage of unchanged carotene.

Since the strongest evidence in favour of the conversion must be derived from those cases in which carotene of the highest purity was used, special attention may be first directed to rats nos. 15 to 18 which received carotene (M.P. 178° uncor.) which had been obtained from carrots and recrystallised twelve times from cyclohexane, operations being conducted for the most part in an atmosphere of nitrogen. The pigment was added in cyclohexane to the arachis oil used in compounding the basal diet, the cyclohexane being removed under diminished pressure. After having attained the "plateau" condition each rat received the carotene during from 16 to 26 days at the rate of 0·75 mg. per 10 g. of diet eaten, which was the average amount consumed daily. At autopsy, despite very liberal amounts of pigment ingested, the body fat was always found to be quite colourless, apart from occasional small loci in regions where the intra-peritoneal fat lay in contact with the gut wall. The liver oils were certainly yellower than the liver oils of the control rats which had received no carotene, but these increases in yellow values were trivial when contrasted with the enormous concentration of yellow pigmentation that would have been expected if the intense blue colours now given in the $SbCl_3$ reaction had been due to unchanged carotene. The following observations confirm the view that conversion had been effected. (1) These blue colours were now characterised in all cases by a well-defined band at 610–630$\mu\mu$, the position characteristic of vitamin A, and not at 590$\mu\mu$, the position characteristic of the ingested carotene. (2) In preliminary work by Mr N. S. Capper (private communication) it was found that the untreated liver oil now displayed the absorption band at 320–330$\mu\mu$ characteristic of vitamin A. (3) As a final precaution against the possibility that the chromogen should represent an inactive degeneration product of the pigment the vitamin A activity of specimen liver oils was confirmed by biological tests (Fig. 2).

In regard to rats nos. 18 to 22 which received even more liberal supplies of carotene as red palm oil or carrots, similar results were obtained, in which the yellow and blue values of the liver oils attained somewhat higher levels. For example the liver of rat no. 21, which had received during several weeks a diet consisting largely of carrots, was bright orange in colour. Such obvious pigmentation might at first sight be taken to support the claim of Euler, Euler and Karrer that the carotene had been stored in an unchanged condition, but it may be pointed out that the blue value of the liver oil would have corresponded to the presence of about 80 mg. of the pigment, *i.e.* the liver oil would have appeared deep crimson and would have deposited crystalline carotene freely on standing.

Expression of results.

The colorimetric examination of the liver oils was carried out according to the technique previously described [Moore, 1929, 2]. The method of expressing results may be explained most simply by an actual example.

Rat no. 18. Liver oil dissolved in 10 cc. chloroform.

Yellow units. The colour of this solution was matched by Lovibond glasses 4 yellow, 0·7 red. Therefore total number of yellow units in liver = 4 × 10 (volume of solution in cc.) = 40 units.

Blue units. 0·05 cc. of the above solution was made up with washings of chloroform to 0·5 cc. $SbCl_3$ reagent (2 cc.) was then added. The resulting blue colour was matched by Lovibond glasses 4·0 blue, 1·0 yellow (average of several readings). Therefore total number of blue units

$$= 4{\cdot}0 \times \frac{2{\cdot}5 \text{ (volume of reaction mixture)}}{0{\cdot}05 \text{ (volume of solution used in test)}} \times 10 \text{ (volume of the original solution)}$$

= 2000 units.

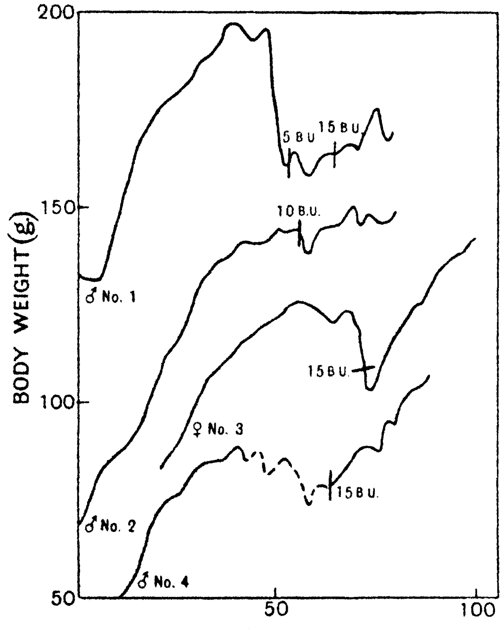

Fig. 2. The vitamin A activity of the liver oils of rats (no. 15 and 18 of Fig. 1) which had received excess of carotene.

Doses are expressed in Lovibond blue (" 1 cm. cube ") units. Rat no. 3 was completely cured of severe xerophthalmia and a septic cheek. N.B. Rat no. 4 received an ineffective material in a separate experiment during the period indicated by the broken curve.

It should be noted that the yellow and blue values so expressed are directly comparable. The method of calculation will be seen to be exactly the same in each case when it is remembered that the addition of the $SbCl_3$ reagent in obtaining the blue value necessarily involves a further dilution of the chromogen.

Table I. *Analyses of liver oils for vitamin A and carotene.*

Rat no. and sex	Body wts.: initial; plateau; final (g.)	No. of days on vitamin A-free diet	Dosage of carotene (mg.)	No. of days of carotene feeding	Total Lovibond units ingested by rat		Total Lovibond units found in liver		Total yellow units to be expected in liver if blue chromogen were carotene
					Blue units in $SbCl_3$ reagent at 590$\mu\mu$	Yellow units in $CHCl_3$	Blue units in $SbCl_3$ reagent at 610–630$\mu\mu$	Yellow units in $CHCl_3$	
			Rats receiving vitamin A-free diet. No treatment.						
1 ♂	54, 171, 124 (D)	77	—	—	—	—	0	4	—
2 ♂	41, 121, 105 (K)	67	—	—	—	—	0	9	—
3 ♂	45, 105, 89 (K)	67	—	—	—	—	0	5	—
4 ♀	42, 119, 109 (K)	51	—	—	—	—	0	6	—
5 ♂	54, 99, 92 (D)	33	—	—	—	—	0	2	—
6 ♀	31, 58, 58 (D)	26	—	—	—	—	0	2	—
7 ♀	33, 60, 45 (D)	39	—	—	—	—	0	1	—
8 ♀	85, 127, 105 (K)	54	—	—	—	—	0	5	—
9 ♂	140, 167, 145 (D)	50	—	—	—	—	0	10	—
10 ♀	62, 100, 77 (D)	54	—	—	—	—	0	3	—
			Rats cured by small doses of carotene (M.P. 174°).						
11 ♀	45, 111, 133 (K)	54	0·02	20	70	770	0	10	0
12 ♂	51, 128, 178 (K)	47	0·01	43	80	880	0	16	0
13 ♀	52, 114, 133 (K)	53	0·05	45	400	4,400	0	7	0
14 ♀	—, 89, 89 (K)	—	0·5	3	270	3,000	0	23	0
			Rats cured by large doses of carotene (M.P. 178°).						
15* ♂	38, 95, 159 (K)	41	0·75	16	2,200	24,000	2.000	90	22,000
16* ♀	33, 95, 139 (K)	41	0·75	22	3.000	33,000	3,300	110	36,000
17 ♀	36, 66, 106 (K)	29	0·75	26	3,500	39,000	3,700	100	41,000
18 ♂	41, 76, 137 (K)	28	0·75	26	3,500	39,000	2,000	40	22,000
			Rats cured by red palm oil.						
19 ♂	33, 73, 152 (K)	29	R.P.O. 1·5 g.	26	12,000	130,000	5,000	280	55,000
20* ♀	38, 94, 127 (K)	41	R.P.O. 1·5 g.	16	7,400	83,000	4,500	400	50,000
			Rats cured by fresh carrots.						
21 ♀	35, 86, 147 (K)	56	Carrots *ad lib.*	55	?	?	16,000	600	180.000
22 ♂	49, 114, 153 (K)	71	,,	35	?	?	5,300	250	58,000

D = Died. K = Killed.

* These rats were first cured by very minute doses of carotene before the larger amounts were given.

In calculating the colour value of the ingested pigment in Table I the values for both samples of carotene have been taken as blue = 180 units per mg. per cc., yellow = 2000 units per mg. per cc.

DISCUSSION.

The above results afford a complete picture of the conversion of carotene *in vivo* to the familiar vitamin A of liver oils, as defined by all the chromogenic and spectroscopic properties which have been found to run parallel to this factor. The details of the conversion may be summarised as follows:

Carotene	⟶	**Vitamin A**
Synthesised in plant		**Stored in animal**
Intensely yellow		**Almost colourless**
328$\mu\mu$ absorption band absent		**328$\mu\mu$ absorption band developed**
Greenish blue $SbCl_3$ reaction at 590$\mu\mu$		**Vivid blue $SbCl_3$ reaction at 610–630$\mu\mu$**

This evidence could be regarded as conclusive if it could be stated with certainty that the carotene used was completely pure. Upon this point, however, doubts might be raised, since Euler, Karrer and Rydbom [1929] have advanced certain evidence, based upon supposed variations in the activity of different samples of carotene, which raises the question as to whether the activity of the pigment may not after all be due to the presence of a "stubbornly adhering" impurity active at about the same level as vitamin D. The danger of this possibility was indeed pointed out by the writer [Moore, 1929, 4] at a time when Euler, Euler and Hellström were satisfied with the chemical purity of their carotene, but it is now obvious that this view in its simplest form would imply a complete rejection of the chromogenic evidence, since it is obvious that the same chromogen cannot be present in both carotene and cod-liver oil. It should be noted that the evidence of Euler, Karrer and Rydbom [1929] is based on small variations in the growth rates of rats and in blue values determined by the tintometer technique. Results obtained by both these methods are notably open to considerable experimental errors, and must therefore be of very doubtful significance.

A point of greater interest, however, arises from the statement of Drummond and Baker [1929] that vitamin A probably constitutes only a minute fraction (10 % and probably much less) of even the most potent liver oil concentrates. Experiments described above (Part V) have shown that the minimal dose of carotene is only slightly lower than that of the most active concentrates, which may actually exceed the pigment in regard to chromogenic activity. From this evidence it might be expected that the activity of carotene would similarly be derived from a small fraction of the total pigment. Two alternative possibilities, however, may be borne in mind, either that the conversion of carotene may be of an inefficient character, or that the estimate of Drummond and Baker may be unduly low. For the present the evidence does not seem of sufficient weight to justify any serious doubt that the physiological activity of carotene is not derived from the pigment *per se*.

SUMMARY.

Part V.

1. The $SbCl_3$ colour reaction given by a minimal physiological dose of carotene at 590 $\mu\mu$ is slightly less than that given by a minimal physiological dose of the vitamin A of liver oil at 610–630 $\mu\mu$.

2. It is therefore impossible that the colour reaction of carotene could conceal any underlying colour reaction due to the liver oil vitamin A in such amounts as would account for its physiological activity.

Part VI.

1. The liver oils of rats suffering from vitamin A deficiency invariably gave negative results when tested with the $SbCl_3$ reagent.

2. Rats after depletion of vitamin A were cured by the administration of large excess of carotene (12 times recrystallised from cyclohexane, M.P. 178° uncor.), red palm oil, or fresh carrots. Traces of yellow pigment now appeared

in the liver oil but the predominant chromogen present (99 %) was invariably vitamin A, as characterised by (*a*) absence of such intense yellow pigmentation as must have accompanied the storage of carotene as such; (*b*) an intensely positive $SbCl_3$ reaction showing a marked band at 610–630$\mu\mu$; (*c*) the development of an absorption band in the untreated oil at 328$\mu\mu$; (*d*) intense biological activity.

3. The conclusion must therefore be reached that carotene, or some part thereof, if it should later prove to be heterogeneous, behaves *in vivo* as a precursor of the vitamin.

My thanks are due to Dr L. J. Harris for his valuable criticism, to Mr T. A. Webster for advice on the extraction of liver oils and to Messrs Chivers and Sons, Limited, for drying the large quantities of carrots from which the carotene used in this experiment was derived. The care of the experimental animals was in the capable hands of Mr A. Ward.

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LXXX. PHOSPHORIC ESTERS IN ALCOHOLIC FERMENTATION.

III. THE LAG BETWEEN PHOSPHATE ESTERIFICATION AND CARBON DIOXIDE EVOLUTION.

IV. OXIDATION-REDUCTION POTENTIALS OF YEAST PREPARATIONS.

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III. The lag between phosphate esterification and carbon dioxide evolution.

Euler and Johansson [1913] were the first to suggest that in the reaction of phosphate with yeast preparations fermenting sugar the phosphate was esterified some time before the equivalent carbon dioxide was liberated. These workers considered that a preliminary activation of sugar, followed by formation of hexosediphosphate and a triosemonophosphate, was responsible for the lag in evolution of gas. In addition to the lag between the reactions of phosphate esterification and carbon dioxide formation there is a delay in the esterification after the phosphate is added. When yeast preparations (particularly maceration extract and preparations containing much water) are added to sugar there is a considerable induction time before any fermentation occurs; the free phosphate present in the preparation then reacts during the period called the "Gäranstieg" by Meyerhof [1918]. This induction period and the "Gäranstieg" caused by the free phosphate inherent in preparations are not discussed in the present work.

In a recent paper on the reaction of phosphate with dried yeast fermenting fructose [Boyland, 1929] a lag similar to that of Euler and Johansson was described. This lag between the processes of esterification and carbon dioxide formation was not due to physical causes such as supersaturation, and it was suggested that it was due to the relatively slow decomposition of products formed after, or simultaneously with, phosphate esterification. The nature of this lag is investigated in the following experiments.

Harden and Young [1908] showed that in the reaction of phosphate with yeast preparations the time necessary for the attainment of the maximum rate of carbon dioxide evolution was much greater with glucose than with fructose, and that this time could be reduced by the addition of relatively small quantities of fructose [Harden and Young, 1909]. Harden and Henley [1920, 1921] found that this time was much reduced by the addition of hydrogen acceptors such as methylene blue, pyruvates or aldehydes. Acetaldehyde was the most active agent in this way, being about fifty times as efficacious as fructose. Under almost all conditions the reaction of one molecule of phosphate in fermentation yields one molecule of carbon dioxide, whether monophosphoric or diphosphoric ester is formed. Combining the recent ideas of Harden [1930] with those of Neuberg and Kerb [1913], the sequence of fermentation in presence of a trace of aldehyde might be represented as follows:

$$\begin{array}{lll}
(1) & 3C_6H_{12}O_6 + 2R_2HPO_4 & = 2C_6H_{11}O_5(PO_4R_2) + 2CH_3.CO.CHO + 4H_2O, \\
(2) & 2C_6H_{11}O_5(PO_4R_2) + C_6H_{12}O_6 + 2R_2HPO_4 & = 2C_6H_{10}O_4(PO_4R_2)_2 + 2CH_3.CO.CHO + 4H_2O, \\
(3) & CH_3.CO.CHO + CH_3.CHO & = CH_3.CO.COOH + C_2H_5OH, \\
(4) & CH_3.CO.COOH & = CH_3.CHO + CO_2.
\end{array}$$

Of these reactions the only one likely to be affected by hydrogen acceptors is the oxidation of methylglyoxal to pyruvic acid (equation 3), and as hydrogen acceptors have such a marked effect on carbon dioxide evolution this oxidation is probably the slowest or controlling stage in the beginning of the reaction of phosphate. It should therefore be responsible for the lag in the appearance of carbon dioxide after phosphate esterification and for this reason the effect of glucose, fructose and hydrogen acceptors on the lag was examined.

EXPERIMENTAL.

The preparation used in most of these experiments was the "zymase" free from phosphatase, made by the acetone precipitation of yeast juice, which has previously been described [Boyland, 1930]. It was possible to obtain reliable preparations free from hexosephosphatase by grinding the yeast juice precipitate with half its weight of kieselguhr together with the sugar and water necessary for the fermentation. The whole was then centrifuged and the clear centrifugate used for the fermentation. Harden and Macfarlane [1930] have indicated that hexosephosphatase is probably adsorbed by kieselguhr.

The methods used were largely the same as those of previous experiments, the analytical procedure being identical. When hydrogen acceptors were added, the addition was made 15 minutes before the addition of phosphate. The concentrations of the added substances are calculated on the final volume of the fermenting solution after the addition of phosphate.

EFFECT OF HYDROGEN ACCEPTORS ON THE REACTION.

The experiments of Table I, in which the lag is expressed as the difference between the phosphate esterified and the carbon dioxide evolved at any particular time, show that hydrogen acceptors cause a marked increase in the rate of reaction of phosphate, without diminishing the amount of lag as measured by molecular concentrations. Hydrogen acceptors sometimes increase the lag in amount, though not the period during which it persists; it may be possible

Table I. *The lag between phosphate esterification and carbon dioxide evolution in fermentation with the acetone precipitate of yeast juice.*

In each case 1 cc. 2*M* K_2HPO_4 added to 2 g. yeast preparation in 20 cc. sugar solution.

Results in total millimols.

Time after phosphate addition (mins.)		5	10	20	30	40	50	60	70
Exp. 180									
Glucose alone	P esterified	0·08	0·20	—	0·83	—	1·65	2·00	2·00
	CO_2 evolved	—	0·09	—	0·63	—	1·55	2·10	2·10
	Lag	0·08	0·11	—	0·20	—	0·10	—	—
Exp. 203									
Fructose alone	P esterified	0·10	0·25	—	0·84	—	1·90	2·00	—
	CO_2 evolved	—	0·12	—	0·70	—	1·82	2·06	—
	Lag	0·10	0·13	—	0·14	—	0·08	—	—
Exp. 204									
Fructose with methylene blue (0·05 %)	P esterified	0·12	0·28	—	1·15	—	1·99	1·99	—
	CO_2 evolved	—	0·10	—	0·90	—	1·94	2·03	—
	Lag	0·12	0·18	—	0·25	—	0·05	—	—
Exp. 199									
Fructose with acetaldehyde (0·15 %)	P esterified	0·30	0·80	1·60	1·96	—	—	—	—
	CO_2 evolved	0·16	0·64	1·56	1·97	—	—	—	--
	Lag	0·14	0·16	0·10	—	—	—	—	—

Table II. *The effect of varying concentrations of acetaldehyde on the lag.*

10 cc. 0·6 *M* K_2HPO_4 added to 4 g. dried yeast in 30 cc. 10 % fructose.

Concentrations expressed as molecules per litre.

Concentration acetaldehyde	Change after 20 minutes' reaction		
	P esterified	CO_2 evolved	Difference or lag
Nil	0·017	—	0·017
0·015	0·022	0·009	0·013
0·030	0·036	0·025	0·011
0·045	0·037	0·024	0·013
0·060	0·043	0·024	0·019
0·075	0·042	0·018	0·024

that in the presence of certain concentrations of acetaldehyde, pyruvic acid is formed more quickly than it can be decomposed by the carboxylase, and under such conditions the decarboxylation would become the master reaction. The lag occurs in the reaction with all the yeast preparations examined (acetone precipitate of yeast juice, yeast juice, zymin and dried yeast). It varies in extent with different preparations and must depend upon the balance of enzymes present. It is greater with larger additions of phosphate and it cannot be much reduced by any concentration of acetaldehyde. Table II shows the lag with dried yeast fermentations with varying concentrations of acetaldehyde. The results are also set out in Fig. 1 and the optimum concentration of acetaldehyde is fairly well marked.

As this lag is not removed by hydrogen acceptors—which increase both the rate of esterification and of carbon dioxide evolution—and is fairly constant in amount for any one preparation, it is probable that esterification is normally arrested by accumulation of methylglyoxal. The removal of methylglyoxal (by oxidation at the expense of hydrogen acceptors) allows more sugar to be esterified.

TRIOSE IN THE REACTION.

If the lag in fermentation is due to the relatively slow oxidation of methylglyoxal, then that compound should accumulate in fermentation when the lag is at its greatest. The triose present during the reaction was therefore estimated by the distillation method described by Neuberg, Färber, Levite and Schwenck [1917]. The filtrate from the trichloroacetic acid precipitation was distilled with 10 % sulphuric acid, while water was dropped in to keep the concentration of sulphuric acid constant. All trioses were thus converted into methylglyoxal, which was precipitated from the distillate by heating with a solution of *p*-nitrophenylhydrazine. When cold the precipitate of methylglyoxal *bis*-nitrophenylhydrazone was filtered off in a Gooch crucible, washed with cold water, dried *in vacuo* and weighed. The presence of acetaldehyde does not affect the estimation because the *p*-nitrophenylhydrazone of acetaldehyde is soluble in water in the concentrations in which it would occur in the distillate from fermentations.

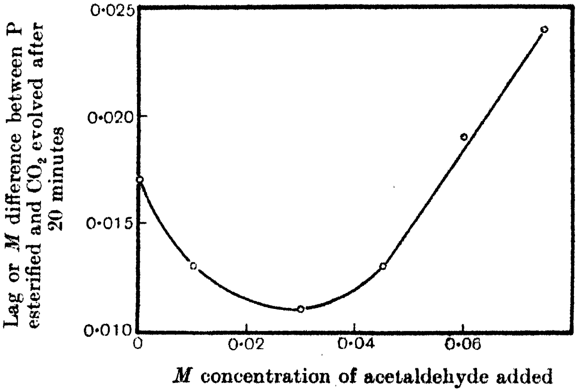

Fig. 1.

That there is an increase in the amount of triose present when the lag in fermentation is at its maximum is shown by the results obtained in Table III. The agreement between the amount of lag and the amount of triose formed is not very exact but the changes are of the same order.

Table III. *The triose present during the period of lag.*

Conditions as in Table I. Results expressed in total millimolecules.

Time after addition of phosphate (mins.)		0	5	10	15	20	30	50
Exp. 180								
Fermenting glucose	Lag	—	0·08	0·11	0·25	—	0·20	0·10
	Triose present	0·16	0·23	0·36	0·34	—	0·25	0·21
	Additional triose formed	—	0·07	0·20	0·18	—	0·09	0·05
Exp. 199								
Fermenting fructose with acetaldehyde	Lag	—	0·14	0·16	0·10	0·04	—	—
	Triose present	0·09	0·19	0·17	0·11	(0·25?)	0·10	—
	Additional triose formed	—	0·10	0·08	0·02	(0·16?)	0·01	—

Although the method of distillation with dilute sulphuric acid yields methylglyoxal in the distillate, methylglyoxal itself does not appear to be present in the trichloroacetic acid filtrates from fermentations. Methylglyoxal is precipitated by dinitrophenylhydrazine in cold acid solution, but the filtrates from fermentations never give the methylglyoxal derivative with this reagent in the cold. If dinitrophenylhydrazine is added immediately after removal of the proteins, a precipitate of acetaldehyde dinitrophenylhydrazone (M.P. 151–152° after recrystallisation from pyridine and ether) is obtained. But, if the trichloroacetic acid filtrate is allowed to stand for more than an hour before the addition of the hydrazine, no precipitate is obtained. Thus even acetaldehyde changes in the solution and it is possible that methylglyoxal, which is still more reactive, changes almost immediately to some compound, possibly a polymer, which is reconverted into methylglyoxal on distillation with acid.

Kluyver and Struyk [1927] suggest that a triosephosphoric ester is formed in fermentation. Such a compound might not be precipitated by hydrazines, yet yield methylglyoxal on distillation with acid. There is, however, no definite support for the existence of such a triosephosphoric ester.

IV. Oxidation-reduction potentials of yeast preparations.

If acetaldehyde acts as an oxidising agent or hydrogen acceptor in fermentation, it must have a definite apparent oxidising potential. It cannot have a true oxidation-reduction potential, because it is reduced irreversibly. The reduction is not a simple electronic change and the reduction product, alcohol, is a feeble reducing agent in aqueous solution. The apparent potential of acetaldehyde was determined by adding it to solutions of leuco-dyes of known oxidation-reduction potential in buffered phosphate solution at p_H 6·0. In this way acetaldehyde was able to oxidise reduced indigodisulphonate, methylene blue and thionine, so that its oxidation potential at p_H 6·0 must be higher than 0·10 volt. Acetaldehyde will not oxidise potassium ferricyanide or the leuco-compounds of 1-naphtholindophenol-2-sulphonic acid and 1-naphtholindo-2:6-dibromophenol-2-sulphonic acid, so that its oxidation potential is probably below 0·15 volt. These determinations were carried out at p_H 6·0 because this was found to be about the p_H of fermenting yeast preparations containing the usual amount of phosphate and saturated with carbon dioxide.

The p_H of fermentation was determined by use of the glass electrode. The reaction of phosphate with yeast juice fermenting fructose was carried out in the cup of a glass electrode similar to that described by Bayliss, Kerridge and Verney [1926]. The readings over 40 minutes during the reaction varied from p_H 6·2 to 6·3. This result is in agreement with the results obtained by Biilmann and Katagiri [1927] for phosphate solutions containing glucose and alcohol and saturated with carbon dioxide. These workers used the quinhydrone electrode, which cannot be applied to yeast solutions on account of their oxidation-reduction potential.

Although glucose is more strongly reducing than fructose at p_H 7·5–9·0 [Wurmser, 1927], in solutions as acid as p_H 6·0 it is doubtful if glucose and fructose have any oxidising or reducing power. In buffered phosphate solution at p_H 6·0 neither glucose nor fructose could reduce methylene blue or oxidise leuco-naphtholindophenoldisulphonate in 12 hours at 30°. It is therefore improbable that the different behaviour of glucose and fructose in fermentation with free phosphate is due to difference in oxidising or reducing properties. The difference in behaviour possibly lies in a difference in the ease of esterification of the two sugars.

Cannan, Cohen and Clark [Clark and collaborators, 1928, p. 306] had found that the oxidation-reduction potential of suspensions of washed yeast cells at p_H 5·75 fell from 0·10 to – 0·05 volt in 5 hours. Having determined that the p_H of yeast preparations remained steady during the reaction of phosphate, it became possible to determine the oxidation-reduction potential by adding dyes, and noting the time necessary for decoloration. The acetone precipitate of yeast juice, being free from hexosephosphatase, was very useful for this determination of the oxidation-reduction potential of fermentation with free phosphate, because no fermentation or reduction of dyes occurred until free phosphate was added. The time taken for the reduction of various dyes added in minimal concentrations (0·01 %), so as to have as little poising effect as possible, are shown in Table IV. The figures for the approximate E_0' (the E_h at which the dyes are 50 % reduced) at p_H 6·0 are taken from papers by Clark and his collaborators [1928]. The results show that the oxidation-reduction potential falls from 0·10 to – 0·03 while the greater part of the reaction with phosphate occurs.

Table IV. *The time taken for the reduction of dyes added to the acetone precipitate of yeast juice fermenting glucose in the presence of* K_2HPO_4.

Dye added (0·01 %)	E_0' at p_H 6·0	E_h for 98 % reduction	Time for reduction (mins.)
1-Naphtholindo-2:6-dibromophenol-2-sulphonate	0·19	0·14	2
1-Naphtholindophenol-2-sulphonate	0·18	0·13	5
Thionine	0·10	0·05	10
Methylene blue	0·05	0·00	15
Indigotetrasulphonate	0·00	– 0·05	30
Indigotrisulphonate	– 0·03	– 0·08	Partially reduced
Indigodisulphonate	– 0·07	– 0·12	Not reduced

Experiments were next carried out with higher concentrations of the dyes so that they exerted some poising effect on the system. In this way the rate of fermentation in the presence of free phosphate was measured at different oxidation-reduction potentials. Results of such experiments are shown in Table V. It will be seen that dyes more strongly oxidising than thionine or more strongly reducing than indigodisulphonate do not accelerate the fermentation, but in addition to methylene blue both thionine and indigotetrasulphonate act as hydrogen acceptors. The failure of indophenol dyes to increase fermentation may be due to their quinone structure, so that they might combine with the protein of the enzyme, or to the phenolic structure of their reduced forms so that they might inhibit fermentation in the same way as phenol. Both quinone and quinol hinder fermentation. The dyes, however, do not seem to combine in any way, as on addition of potassium ferricyanide at the end of fermentation the colour of the dye is always restored. Potassium ferricyanide, a different type of compound and a stronger oxidant than any of the dyes used, is reduced in fermentation but the fermentation is not increased.

Table V. *The effect of different hydrogen acceptors and reducing agents on fermentation.*

1 cc. $2M$ K_2HPO_4 added to 2 g. acetone precipitate of yeast juice in 20 cc. 10 % glucose solution.

Substance added	E_h during reaction (approximate)	Millimolecules CO_2 evolved in 20 minutes
None	0·00?	0·16
0·05 % indigodisulphonate	– 0·03	0·10
0·05 % indigotrisulphonate	– 0·02	0·10
0·05 % indigotetrasulphonate	– 0·01	0·52
0·05 % methylene blue	0·01	0·47
0·05 % thionine	0·03	0·40
0·05 % naphtholindo-2:6-dibromophenol-2-sulphonate	0·14	0·18
0·05 % naphtholindophenol-2-sulphonate	0·13	0·10
0·05 % acetaldehyde	—	0·86
Leuco-dyes		
0·05 % leuco-indigodisulphonate	– 0·10	0·08
0·05 % leuco-methylene blue	0·00	0·42
0·05 % leuco-naphtholindophenol-2-sulphonate ...	0·12	0·12

The effect of adding leuco-dyes throws some light on the mode of action of dyestuffs in fermentation. Leuco-indigodisulphonate almost stops fermentation, but leuco-methylene blue accelerates fermentation to nearly the same extent as the oxidised dye and leuco-naphtholindophenolsulphonate has no effect on the fermentation. As reduction does not therefore have any great effect on the action of dyes, it is probable that they act as hydrogen carriers rather than as hydrogen acceptors. Thus it might be expected that methylene blue would be reduced by methylglyoxal and oxidised again by acetaldehyde (the last reaction can be shown to take place in the test-tube without enzyme). If this is the case, only those dyes which can be oxidised by acetaldehyde and reduced by methylglyoxal will be active in fermentation and the apparent reducing potential of the active methylglyoxal of fermentation should be about – 0·02 volt at p_H 6·0.

The action of acetaldehyde in accelerating the reaction of phosphate is probably different from that of dyes. The reduction of aldehyde differs from that of the dyes which accelerate fermentation in not being reversible, so that acetaldehyde acts purely as hydrogen donator, not as hydrogen carrier. It is probably more effective in fermentation because of some specificity of the oxidase for acetaldehyde.

SUMMARY.

1. The lag between phosphate esterification and carbon dioxide evolution, in fermentation by yeast preparations, is not removed by the addition of those hydrogen acceptors which reduce the time necessary for the attainment of the maximum phosphate rate of carbon dioxide production.

2. The lag is accompanied by an increase in the amount of triose present; it is possible that accumulation of methylglyoxal prevents further esterification of phosphate.

3. During the reaction of phosphate in fermentation the p_H of the solution, saturated with carbon dioxide, remains constant between 6·2 and 6·3.

4. Those dyes which accelerate fermentation by acting as hydrogen acceptors or carriers have an E_0' between 0·00 and 0·10 volt at p_H 6·0; it is probable that they must be reducible by methylglyoxal and oxidised again by acetaldehyde.

My best thanks are due to Prof. A. Harden for much helpful criticism, and to Dr Winifred Pleass, who carried out the p_H determinations with the glass electrode.

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LXXXI. CARBOHYDRATE METABOLISM IN BIRDS.

II. BRAIN LOCALISATION OF LACTIC ACIDOSIS IN AVITAMINOSIS B, AND ITS RELATION TO THE ORIGIN OF SYMPTOMS.

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In a recent paper [Kinnersley and Peters, 1929] it was shown that opisthotonus symptoms due to avitaminosis B_1 in pigeons were associated with an increase of lactic acid in the brain. The provisional conclusion was reached that the increased lactic acid was specifically due to lack of vitamin B_1 in the brain. If this conclusion be true, not only does it provide cogent evidence for the relation between vitamin B_1 and some phase of carbohydrate metabolism, but we feel that it is evidence of chemical change underlying the activity of the central nervous system. We have therefore analysed the problem further. It was conceivable that the changes in the brain were merely due to a general increase of lactic acid throughout the tissues. If this compound was present especially in one region of the brain, it would show that there was no direct relation between the increased blood-lactic acid and that in the brain. Further, it would be suggestive evidence for a correlation of the symptoms with a localised chemical abnormality and would indicate the portion of the brain responsible for them. Some preliminary experiments upon this question have already been published [Kinnersley and Peters, 1930].

EXPERIMENTAL.

The treatment of the brain tissue and the methods of analysis have been described elsewhere. No significant changes have been made. Use has been made of the "guillotine" method. The two halves of the brain have been immersed in liquid air within 5 seconds approximately, the time of immersion being stated in the tables. Freezing, though instantaneous at the cut surface, must take some seconds for completion throughout the mass. When removed from the liquid air, the brains are too hard to divide. Fortunately, we have found that thawing produces little change, if any, in brain tissue which has been frozen in liquid air. We have previously quoted two experiments in support of this [Peters, 1930]. Further experiments which illustrate the same point are shown in Table I, Exps. 1, 2 and 7. Allowing the brain tissue to thaw completely has not led to large increases in lactic acid content. In one case (Exp. 7) there would seem even to have been a decrease of the lactic acid. We do not, however, lay stress upon these changes. They indicate that brain tissue can probably be allowed to thaw completely without much change in content of lactic acid. In practice, the required parts of the tissue have been removed before being completely thawed, to minimise any possibility of change in the abnormal tissues. About 15–20 minutes after removal from the liquid air the tissue can be cut with a knife. At this stage portions were removed with a clean, cooled scalpel and dropped into ice-cold 10 % trichloroacetic acid; any congealed blood was cleaned from the split half of the brain before selecting the parts. As the significance of the experiments depends upon comparative rather than upon absolute values, great care has been taken to work under strictly comparable conditions throughout.

Parts of the brain selected. These have been in various experiments: (1) the cerebrum, (2) the cerebellum, (3) the mid-brain with the optic lobes, and (4) the remainder, bulb and medulla. Various combinations of these have been used. By cerebrum we mean here the parts of the brain included by a horizontal cut parallel with the lower surface of the cerebral hemispheres, and in the sections of mid-brain have been included the optic lobes together with such tissues as lie in the median plane upon a horizontal line drawn approximately parallel with the lower surface of the optic lobes. The approximate weights are for the cerebrum 0·96 g., cerebellum 0·27 g., mid-brain and the remainder 0·43 g.

"*Sugar values.*" These have been obtained upon blood by the Hagedorn and Jensen method, using zinc precipitation as described by these authors. Values for "sugar" by the Hagedorn and Jensen method, obtained upon trichloroacetic acid filtrates, are usually higher than these, but are not consistently so. As shown recently by Gulland and Peters [1930], these differences are due to varying amounts of glutathione and are not so reliable as the direct zinc precipitation. In any case, values would have to be reduced by some 70 mg. per 100 cc. to obtain the true "sugar" value.

Determination of lactic acid. The following is a description of the method actually used. The samples of brain tissue were placed in tared 25 cc. centrifuge tubes containing about 5 cc. of 10 % ice-cold trichloroacetic acid to 1 g. of tissue; the tubes were quickly re-weighed to obtain the weight of the tissue. It was minced with a glass rod and left to extract in the trichloroacetic acid with the addition of an equal volume of water for 30 minutes or longer and was then centrifuged and filtered. Fresh trichloroacetic acid (2–3 cc.) was added to the residue, which was ground and allowed to stand for re-extraction for at least 30 minutes. The latter operation was repeated. Water was added and the tube centrifuged. The combined centrifugates made a total volume of about 20 cc. It was not necessary to leave the tissue to stand for 24 hours as has been claimed recently by Jungmann and Kimmelstiel [1929]. The combined centrifugates were divided into two (or in later experiments into three) parts and each of these was separately treated with 1 cc. of 10 % copper sulphate and sufficient $Ca(OH)_2$ suspension to make it alkaline (about 4 cc.). By performing these extractions separately, it has been our belief that a more proper control is made of the errors produced in the copper-lime treatment. After standing, the mixtures were centrifuged, and the clear supernatant fluid was used for determination of lactic acid by the method of Friedemann, Cotonio and Shaffer [1927].

Accuracy of the lactic acid determinations. In the case of blood, where determinations have been made upon trichloroacetic acid filtrates, duplicate values have always agreed remarkably closely, to within ± 0·01 cc. of $N/100$ iodine. This means that the values given in the tables are the average of two samples not differing from one another by more than ± 0·5 mg./100 g. tissue. For the brain tissue, all the values given in the tables are the mean of two duplicates which did not differ by more than ± 0·02 cc. (approximately ± 0·01 mg. lactic acid) and were often identical. Occasionally we have found quite wide and inexplicable variations between duplicates carried out by our method of separate precipitation at the copper-lime stage, which we are satisfied are not introduced by the reagents. In order to make clear the maximum error allowed in the calculations, it may be said that with the larger amounts of tissue, *i.e.* estimations of cerebrum and cerebellum taken together, or upon the remainder, the values given in the tables will lie within ± 2 mg./100 g. tissue of the figures actually obtained. More generally the variation is much less. But where smaller amounts of tissue only were available the error is proportionately greater.

The method of Friedemann, Cotonio and Shaffer in our hands gave a blank value upon the reagents of 0·05 to 0·06 cc. $N/100$ iodine, and a yield of 95 to 97 % when tested against recrystallised zinc lactate. Lactic acid values in the tables have not been corrected for this difference [see Friedemann, 1928].

The substance estimated. The amounts of lactic acid concerned are too small to estimate directly as the zinc salt; there is in any event doubt as to whether isolation of a zinc salt can be relied upon more than the latest oxidation methods [see Friedemann, 1928]. We have made the assumption that the substance producing aldehyde is lactic acid. Justification for this assumption can hardly be questioned in the case of the normal brains. Lactic acid itself was isolated from human brain many years ago by Thudichum [1884] and McGinty and Gesell [1925] have isolated lactic acid from the brains of dogs. It was possible, however, that the abnormal brains contained some substance other than lactic acid yielding aldehyde. It would be evidence against this if it could be shown that the aldehyde-producing substance was ether-soluble. The following experiment was therefore carried out. The brains of avitaminous pigeons were taken soon after death, extracted as usual with trichloroacetic acid, and an aliquot sample analysed after treatment with copper-lime. The remainder was then saturated with NaCl, 0·5 cc. of conc. H_2SO_4 added, and the whole extracted with ether (specially purified) in a Clausen extractor. The ether filtrates were treated with 10 cc. water, powdered talcum was added and NaOH to alkalinity, and the ether removed upon the water-bath. Portions were taken for direct estimation of lactic acid and also after copper-lime treatment. Under these conditions extraction with ether is slow.

Details of experiment. Two avitaminous brains taken; weight 4·12 g. Trichloroacetic acid extracts analysed. (*A*.)

	(mg.)	(mg.)
Lactic acid present initially in A...		2·25
„ in first extraction with ether 3 hours	1·88	
„ in second extraction with ether 3 hours	0·22	
„ still present in aqueous phase after the extractions	0·14	
Total ...		2·24

In the two ether extractions therefore at least 95 % of the substance estimated was found to be ether-soluble. The excellent balance obtained shows that nothing was added in the process of extraction. This experiment makes it very probable indeed that the substance concerned in the abnormal brain is actually lactic acid [Friedemann and Kendall, 1929].

Normal brains. Table I gives the results for normal birds. Exps. 1 and 2 are the averaged results for two separate groups of five birds. The remainder represent determinations upon single birds. The lactic acid found within the time stated is approximately the same for the cerebrum, cerebellum and the rest of the brain, and amounts to some 50–60 mg. per 100 g. of tissue except in the case of the damaged brain (Exp. 7 (*a*)).

Reducing values were also obtained and showed in each case a diminution for the (*b*) sample as compared with the (*a*), but this varied from 9 to 40 mg./100 g. Throughout the work reducing values have been obtained, but they are omitted here because they show no consistency and add nothing to those previously published. The figures for brain tissue represent mg. of lactic acid per 100 g.; for blood, mg. of lactic acid and sugar per 100 cc.

Table I. *Normal birds.*

Time means interval between death of the brain and immersion of the material in liquid air

Exp.	Time (secs.)	Cerebrum	Cerebellum	Rest	Notes
1	(*a*) 11 (*b*) 83	56 93	46 85	55 92	5 birds used. One-half (*a*) of each brain dropped into liquid air at 11 secs., and the other (*b*) at 83 secs. (*a*) and (*b*) averaged results for each set of half brains
2	(*a*) 11 (*b*) 86	52 87	53 94	58 85	5 birds used as in Exp. 1, but the brains allowed to thaw in air for 30 mins. longer than Exp. 1

Exp.	Time (secs.)	Cerebrum and cerebellum	Rest	Blood- lactic acid	Blood- sugar	Notes
3	5	39	43	14	251	
4	4½	56	56	17	210	
5	4	57	59·5	24·5	233	
6	4½	54	51	25	184	
7	(*a*) 5½	70*	64	33·5	221	*Brain badly damaged by knife
	(*b*)	57	53	—	—	
8	3½	54	58	13	242	"Urethanised"

Our standard technique therefore gives for normal brains similar values for lactic acid in different parts of the same brain.

Opisthotonus brains. Table II shows that in pigeons suffering from opisthotonus due to vitamin B_1 deficiency, there is an increase of lactic acid in the lower parts of the brain as compared with the upper. The deficiency was induced by rice feeding by the usual methods employed in this laboratory.

Table II. *Opisthotonus brains. Acid mg./100 g.*

Exp.	Time (secs.)	Cerebrum	Cerebellum	Rest		Blood-lactic acid	Blood-sugar	Hours symptoms
1	6½	90	94*	124		170	164	?
2	6	65	64*	108		54	357	4
3	6	113	80*	157		—	336	6
4	4	80	85	104		31	246	1½
5	6	87·5	92	137		38	231	1½
		Cerebrum and cerebellum						
6	4–5	63		—		65	283	?
7	5	52*		86		65	405	3
8	5	75		104*		161	177	5
9	5	99		86		53	212	24†
10	7	100		123		62	262	?
11	4	103		113		66	226	2
12	4	89		98		41	270	—
				Mid-brain	Rest			
13	4	61*		52	71*	23	284	2+
14	6	98		114*	124	86	343	—
15	5	76*		105	—	52	—	4
16	4	107		127	113	84	307	—
17	3½	74		87*	98	52	231	2+

* One estimation only.

† Part of mid-brain included accidentally in cerebrum.

Last column indicates the number of hours during which symptoms had persisted.

With one exception, viz. Exp. 13, the blood-lactic acid is high and there is a general tendency for the brain-lactic acid to follow it, though this is not the absolute rule (Exps. 1 and 8). There is only one case, Exp. 9, in which the cerebrum and cerebellum contain more lactic acid than other parts of the brain; in this it was known that some of the mid-brain was accidentally included in the cerebral values. Exps. 13–17 show that as a general rule there is more lactic acid in the lowest section of the brain analysed. The localisation of this acid and its uneven distribution are suggestive evidence that the acid found does not merely accumulate from the blood stream.

In attempting to correlate the "lactic" acidosis with the origin of the symptoms, we examined the condition of the brains in birds with threatening or incompletely developed symptoms. In our previous paper [Kinnersley and Peters, 1929] we showed that rice-fed birds had a normal brain-lactic acid even after some 20 days upon the diet. In one case, however, which could be presumed to be approaching symptoms as it had been upon the diet longer than the others (27 days) we found actually a slight increase. In Table III we have collected a series of results upon birds which in our opinion were threatened with symptoms. The results are somewhat variable as would be expected. One reason for this is that in spite of an extensive experience, it is not easy to be certain in every case how near a bird is to "opisthotonus."

Table III. *Threatening opisthotonus.*

Exp.	Days on diet	Time (secs.)	Cerebrum and cerebellum	Mid-brain	Rest	Blood-lactic acid	Blood-sugar
1	20	3–4	69		79	45	279
2	24	4	—		75	44	234
3	17	5	64		72	67	246
4	22	—	41		72	67	292
5	27	7	62		97	78	269
6	30	4–5	57		73	—	—
7	32	6–7	55		63	21	242
8	12	4	66		96	87	260
9	—	5	48	52*	60*	14	244

* One estimation only.

The results are within normal limits for cerebrum and cerebellum, but raised for the lower part of the brain. If we exclude the two experiments showing a value for the lactic acid in the "rest" as high as 97 mg., the remaining values all lie under 80 mg. per 100 g. Practically all therefore are below those of Table II. Exps. 7 and 8 are within the normal limits for lactic acid in blood and brain. It is very probable that we had here selected birds which were not sufficiently advanced to show the brain condition. This is supported by the low blood-lactic acid. The facts are therefore that in the avitaminous bird, lactic acid begins to increase in the lower parts of the brain before the symptoms of opisthotonus appear. When it reaches a certain value (under the conditions of our experiments some 80 mg. per 100 g.) opisthotonus tends to arise.

The presence of opisthotonus is associated with an increase in the amount of lactic acid present; such increases are again unevenly distributed. A rise in lactic acid usually takes place in all parts of the brain, but this is most marked in the lower regions. The rise in the cerebellum is not more than that in the cerebrum. In the experiments in which the mid-brain and "rest" have been analysed separately, there tends to be more in the "rest," but this is not without exception, though not too much weight can be attached to such differences as appear owing to the experimental error involved in handling these small amounts of tissue. Together with the uneven increases in the brain, there occurs an increase in lactic acid in the blood. The increases in brain-lactic acid, however, do not follow the increases in blood-lactic acid directly. This suggests that the two are really independent, which would also follow from the uneven distribution of the lactic acid in the brain. Before, however, being able to decide that this is so, another possibility must be considered. It has been suggested to us that the uneven distribution of lactic acid might be merely the result of some structural peculiarities in the brain tissue, which lead to irregular adsorption from an increased blood-lactic acid, or to irregular elimination of lactic acid from the tissue. As we have had no success with attempts to raise the blood-lactic acid by intravenous injections of lactic solutions in normal, unanaesthetised birds, we have had recourse to the expedient of flying birds round a room for periods of 30 minutes, and then taking the brains for analysis (Table IV).

Table IV. *Exercised birds.*

				Blood-	
Exp.	Time (secs.)	Cerebrum and cerebellum	Rest	lactic acid	sugar
1	5	62	60	80	237
2	4-5	71·4	73	50	237
3	4	93*	89*	121	184
4	3½	73	79·5	126	219
5	4	81	83	86·5	295

* Poor duplicates.

Table IV brings out several points. Exercise increases the blood-lactic acid as would be expected, bringing it in fact to a level even above the amount usually found in avitaminosis. Together with this increase, when the bird is exhausted (Exps. 3, 4 and 5) there appears in the brain an amount of lactic acid as high as that in the bird with symptoms of threatening opisthotonus; but it is evenly distributed. The lactic acid in the brain does not increase above 90 mg. even with enormously high blood-lactic acid. The localised nature of the chemical lesion in vitamin B_1 deficiency responsible for the appearance of increased lactic acid in certain parts of the brain lends support to the conclusions of Fisher and Peters [1930] that the condition is not caused by impairment of brain circulation. It is associated with the initiation of symptoms of a peculiar type which are cured by administration of vitamin B_1, concurrently with the return of brain-lactic acid to a more normal value. In our opinion the results indicate that there is some missing factor in the tissue. Since this change is ameliorated by giving vitamin B_1 (most speedily of all by the intracranial injection of torulin [Peters, 1930]), lack of vitamin B_1 in the tissue leads to the accumulation of lactic acid in that tissue. This appears to be therefore a proof of the connection of vitamin B_1 with the intermediary metabolism of carbohydrates, a view which has been often mooted [Braddon and Cooper, 1914; Funk and v. Schönborn, 1914; Randoin and Lecoq, 1927].

We are upon less certain ground when we attempt to guess the exact function of torulin from the presence of the excess lactic acid. There is no evidence that the glycolytic power of the tissue is impaired, or that the powers of synthesis of the lactic acid precursor are diminished. In experiments upon birds showing symptoms, we have found that the brain-lactic acid maximum, after allowing the tissue to stand for 90 seconds before freezing, was in one case for cerebrum and cerebellum 142 mg. and for the remainder 150 mg., the blood-lactic acid being 56 mg., and in another case for cerebrum, etc. 178 mg., and for the rest 230 mg. The lactic acid maxima are much greater than normal and are not necessarily different for different parts of the brain in the deficient birds. There must be therefore more precursor in these brains than in the normal. This is curious and as yet unexplained. Insulin lowers the lactic acid maximum for brain and vitamin B_1 deficiency raises it. These facts must be reconciled with any general theory of the condition. The evidence against the view that the increased lactic acid is due to an enhanced rate of glycolysis which has been presented elsewhere is still valid. Upon these grounds we think that vitamin B_1 is concerned with the oxidative removal of lactic acid, though admitting that a substantive proof of this is at present lacking.

We are aware for instance that the appearance of the acid might be secondary to some change in p_H of the tissue induced by lack of vitamin, or that the function of vitamin B_1 might even be to stimulate the production of some missing hormone, which then activates the tissue. In the latter case it seems to be more difficult to explain the localisation.

It must be emphasised that the figures in the above tables, though strictly comparable, do not represent the initial state of the "resting" brain-lactic acid.

Further considerations. In the above experiments, the use of anaesthetics or similar substances has been avoided in order to minimise any complication produced by their results. It must not be forgotten that substances like urethane are well known to disturb surface reactions, and that they inhibit the action of the cell dehydrogenases [Keilin, 1929]. However, since Cobet [1929], in work which only came to our notice when the major part of this research was completed, has found that brain-lactic acid in deep urethane anaesthesia is very low, we have carried out a few experiments upon this point. Cobet found that in the case of rabbits the resting values for lactic acid (by a colorimetric method) were 10–12 mg./100 g., increasing very much upon injury. The pigeons employed by us were deeply urethanised. After having been left undisturbed for periods of 30 minutes, the birds were guillotined and the samples of tissue frozen as usual. Table V gives the results.

Table V. *Lactic acid in brains of "urethanised" animals.*

						Blood-	
Exp.	Condition	Time (secs.)	Cerebrum and cerebellum		Rest	lactic acid	sugar
1	Normal	3½	54		58	13	242
2	,,	3½	46		60	18	237
3	,,	3½	80		73	11	228
4	,,	3½	52		68	13	274
5	Opisthotonus	5–6	82		101	40	249
			Cerebrum	Cerebellum			
6	,,	4	64	80	87	14	253
7	,,	4	72	62	91	26	261

Our "urethane" results in no case approach those of Cobet. It seems to be impossible even with rapid work in pigeons to reach his low limits, though the degree of injury was certainly no more in our experiments. Inspection of Table V shows that the normal urethanised pigeons show most inconsistent values for brain-lactic acid, but have a low blood-lactic acid. Results for avitaminous birds are the same as usual.

Iodoacetic acid. In a recent publication Lundsgaard [1930] has shown that animals poisoned with monoiodoacetic acid die in *rigor* without showing any rise of lactic acid in the muscles. The muscle is fixed as it were in the resting state [see also Meyerhof, 1930]. Table VI shows the amounts of lactic acid found in pigeons killed by intravenous injections of the poison. Injections were made into the wing vein and varied from 50 to 100 mg. of the compound. Brains were removed in the usual manner and immersed in liquid air as soon as possible after the death of the animal, which took place at times varying from 2 to 15 minutes after injection. The time elapsing between injection and death apparently did not influence results.

Table VI. *Pigeons killed by iodoacetic acid.*

Exp.	Condition	Time of death (mins.)	Cerebrum and cerebellum	Rest	Blood-lactic acid	Blood-glucose
1	Normal	4	24·5	24·7	7·9	217
2	,,	8	29·5	29·5	6·6	214
	,,	2	27·5	35	—	—
3	H.R.	5	34	47	26·6	214
4	,,	15	36	56·5	81	231
5	,,	7½	53	75	81	272

These results are of great interest, being the lowest which we have obtained for normal and avitaminous pigeons' brains. It will be noticed that the "opisthotonus" birds all show higher lactic acid, and that in these the lower part of the brain is again 13–22 mg. higher than the values for the cerebrum and cerebellum. These amounts bear roughly the same relation to one another as do those of Tables I and II. Until more evidence is forthcoming as to the exact action of monoiodoacetic acid, we cannot draw conclusions as to whether the experiments of Table VI represent the lactic acid content of the tissues during life. But the results constitute the strongest additional support for the views put forward in the earlier part of this paper, and exclude the possibility that the high lactic acid of the avitaminous brain is due to increased rate of glycolysis.

Relation of the symptoms to the presence of lactic acid.

If the experiments in Tables II and III are examined, it will be noticed that in practically every case in which the cerebrum and cerebellum were separately analysed, the values were identical within the limits of experimental error. They were also always lower than the value for the rest of the brain. In Exp. 2, Table II, the values for cerebrum and cerebellum are actually within normal limits. In the experiments of Table III the fact that often immediately before symptoms appear the values for cerebrum and cerebellum analysed together are within normal limits indicates that the cerebellum is sometimes normal (so far as lactic acid is concerned). This suggests that the symptoms are associated directly with the presence of lactic acid in the lower parts of the brain. Certain other facts support this. Fisher and one of us in another connection have repeatedly removed the cerebrum and basal ganglia under ether anaethesia in avitaminous birds. As soon as the bird recovers from the anaesthetic, the symptoms reappear. They cannot be due to release of cerebral control, because it is well known that decerebration alone makes little difference to the appearance of a pigeon. This confines the problem to the lower part of the brain, and to the cerebellum. Now the removal of the latter induces a similar condition to the "opisthotonus" of avitaminosis. The matter has been lately studied carefully by Bremer and Ley [1927].

Symptoms are induced by removal of the cerebellum, and hence the problem of their origin in avitaminosis reduces either to (1) increased stimulation of the mid-brain, etc., or (2) to release of cerebellar control. Since we have found cases in which symptoms are present with a practically normal "cerebellar"-lactic acid, any possible release of cerebellar control cannot take place by a lactic acid block in the cerebellum. So the symptoms must be associated with increased lactic acid in the lower parts of the brain. The condition produced would appear to be the "brain" analogue of *rigor* in the muscle. Activity of the brain tissue concerned produces lactic acid which then tends to accumulate because it is incompletely removed. When a certain concentration is reached, opisthotonus appears. This would be consistent with the common experience that symptoms can be readily induced by exercise when avitaminosis is sufficiently advanced. It is of course impossible upon the evidence here presented to decide whether the lactic acid acts by inhibition or by stimulation.

Visual inspection of the parts especially affected shows that they appear white, whereas the cerebrum appears grey. This difference in appearance may possibly underly the special susceptibility of the lower parts of the pigeon's brain to lactic acidosis. We do not feel that there is at present any justification for thinking that vitamin B_1 deficiency affects conducting rather than cell tissue, because Dr Carleton[1] finds that the whitish-looking mid-brain tissue contains a large proportion of cells. The matter evidently warrants further investigation.

DISCUSSION.

We recognise that the view which has been advanced as to the origin of these symptoms in avitaminosis does not appear to be consistent with our own experiments in Table IV upon birds exhausted by exercise. These certainly show that lactic acid can accumulate without inducing the syndrome in question, but it is felt that a general condition of this nature cannot fairly be compared with a localised affection.

It must be remembered that ether anaesthesia in the avitaminous bird will abolish the symptoms, without altering the changes in the lactic acid. While the condition here investigated is a concomitant of lactic acid accumulation, this is certainly not the case with other similar symptoms. Insulin convulsions in the pigeon (as has been shown by Holmes and Holmes for the rabbit) are associated with a low brain-lactic acid not varying in amount in different parts of the brain. It is impossible to bring into one scheme all the various conditions (apart from surgical lesions) which will induce this condition in the pigeon, without having recourse to some conception such as that of the "Meyerhof cycle" [1926] for carbohydrate metabolism. With the idea, however, that maintenance of the activity of the tissue depends upon a cyclical chemical process, it becomes possible to understand how changes of varying type may result in the same interference with function. This matter has been discussed elsewhere [Peters, 1930].

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[1] Personal communication.

In our previous paper, we drew attention to the discrepancy between our results and the earlier view of Holmes and Holmes [1925, 1926 and 1927] that lactic acid in the brain arises immediately from the blood-sugar. Since then these authors have kindly drawn our attention to a later publication [1928] in which this view is to some extent modified. We feel that the above results can only be interpreted upon the view that there is a precursor of lactic acid in the tissue itself, however much this may in turn be in equilibrium with the blood-sugar.

SUMMARY.

1. The increased lactic acid found in the brains of pigeons showing symptoms of opisthotonus due to avitaminosis B_1 is localised especially in the lower parts of the brain.

2. In the period of rice feeding during which symptoms are threatening, lactic acid is increased only in the lower parts of the brain.

3. The localised character of the chemical lesion proves that vitamin B_1 is associated with the intermediary metabolism of carbohydrate.

4. Symptoms are due to the accumulation of lactic acid in the lower parts of the brain.

5. The lowest lactic acid found in the brain of a normal pigeon was 24 mg. per 100 g. of tissue, after death by injection of monoiodoacetic acid.

6. Severe exercise may cause a rise in the lactic acid content of the brain, which is distributed evenly over the tissue.

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LXXXII. THE METABOLISM OF DEVELOPING SALMON EGGS.

I. THE SIGNIFICANCE OF HATCHING AND THE RÔLE OF WATER IN DEVELOPMENT.

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MATERIAL.

EGGS of the Atlantic salmon (*Salmo salar*) were procured in the eyed stage and maintained in a small hatchery at a temperature of 8 to 10°. Younger stages were not reared in the laboratory, for although many could be kept alive for some weeks after the journey from the hatchery there was no further development. The series of analyses given is that of a group of 5000 eggs which came to the laboratory in the eyed stage and were from one female. Fifty specimens were taken for each test and the adherent moisture removed between sheets of filter-paper. They were then weighed in a dried extraction thimble supported on cotton wool in a tared weighing bottle. This was the wet weight. The eggs and larvae were dried to a constant weight (the dry weight) at 100°.

OBVIOUS CHANGES DURING DEVELOPMENT.

Experts in fish culture recognise several critical periods in the development of salmonid eggs. For a day or two after fertilisation they are hardy and can be moved about and arranged in the hatchery trays without injury; after this they become exceedingly delicate until the appearance of pigmented eyes within the shell. The first period is one of comparatively undifferentiated cell division, the second that of the organogenesis or morphological differentiation. In the eyed stage very few eggs die and much rough handling is not detrimental. About three weeks before hatching there ensues another period of great mortality, in the phase of histogenesis. Many of the embryos become opaque within the shell while the yolk remains transparent. A large number of such larvae have been hatched and examined. Necrosis usually begins as one or more ring-shaped areas in the tail region from which it spreads rapidly until the whole tail has become opaque. At this stage there is no tail circulation, poor vitelline circulation, feebly and irregularly beating heart and very little movement. No larva hatched in such a condition has recovered. A group of larvae in various stages of opacity was hatched and the "case histories" followed in detail. Extracts from two typical examples are given. The embryos began normally to hatch about Jan. 15.

Case 1. Hatched Dec. 29. Physical conditions; dorsal fin sloughed into a large mass of necrosed tissue; adipose fin had dropped off leaving only a jagged edge; two ring-shaped opaque spots, one situated just posterior to the pectoral fin and the other under the adipose fin; heart rate 84 per minute (control heart rate 94); good circulation to all parts of the body. On Dec. 30 the dorsal fin sloughed off and from then on the fish began to recover, until on Jan. 9 there was no trace of the white patches, the heart rate was normal, and the fish very active.

Case 2. Hatched Dec. 30. Physical conditions; whole of tail half way to adipose fin enclosed in a white sheath of necrosed tissue with a large flap hanging loose; a second small patch under the adipose fin; heart rate 69 per minute (control heart rate 84); poor embryonic circulation and no blood supply over distal parts of yolk sac; very little activity. Hatching produced a slight temporary improvement, but after Jan. 2 the heart rate decreased and the general condition became worse until death on Jan. 6. The sudden temporary improvement after artificial hatching has been noted in most cases.

On Jan. 2, 130 typically affected eggs were selected and divided into two groups. Those of one group were hatched artificially by tearing the shell with fine forceps. Both groups were kept under similar conditions to determine the effect of hatching on the fatality of the condition. The final results were the same, 11 out of the 65 surviving in each case. Natural hatching had taken place before the conclusion of the experiment on Jan. 19. It was noticed that death occurred more quickly in the hatched group, due to the immediate attack of the necrosed tissue by the fungus *Saprolegnia ferax*, while the unhatched larvae might live for some days longer protected by the shell. It is believed that under different experimental conditions, using fungus-free water, the hatched group would have shown a larger proportion of survivors.

If bacteria or mycelia could go through the shell, infect the embryo within, and by destruction of the shell produce hatching, an interesting case of symbiosis would be suggested. To test this idea affected larvae and controls were hatched under aseptic conditions and reared in vials at a suitable temperature, protected from atmospheric contamination. Before hatching any possible infection on the outside of the shell was removed by immersion for one minute in 3 % formalin. Experiments determined that at this age an egg is capable of withstanding an immersion of 3 minutes in 3 % formalin without serious injury. It is believed that an exposure to formalin which would remove infection within the egg would also kill the larva. Controls and some of the experimental animals lived until the conclusion of the experiment some 10 weeks after hatching. Those which died were left in the vials and observed, but there was no decomposition except the usual autolytic one, and examinations of living and dead eggs showed no bacteria or fungus. The conclusion is that it is not from external infection that the larvae die within the shell.

It was found that immersion in formalin of an egg 3 weeks before its hatching time, caused it to hatch within a few hours. Scheminzky and Gauster [1924] showed that trout eggs which are continually exposed during development to the action of a weak electric current hatch a week earlier than the controls. The resulting larvae do not differ in any way from the normal. These authors attribute the result to the destruction of the egg capsule, but it may be due to nothing more obscure than a marked stimulation of embryonic activity.

Wintrebert [1912] has demonstrated the secretion of a protease by the trout embryonic ectoderm which dissolves the shell and permits hatching. It is one purpose of this paper to point out that the phenomena associated with the production of this enzyme have a far greater effect on embryonic metabolism than a plain statement of their final result would indicate. It is necessary for the larva to suspend body-building operations and attend to the problem of blazing a trail to the outside world. The effort profoundly modifies embryonic metabolism and it is not until some weeks after hatching that the uneventful course of development is resumed. The dissolving of a capsule so tough that it is broken down only with difficulty in caustic potash solution [Miescher, 1897] is accomplished by a concentrated protease solution in the perivitelline fluid. Variations in the water content and metabolism, and the frequent death of the embryos within the shell are explained by this developmental modification. The region of the embryo which first begins to die lies in the extremities (tip of tail, fins, etc.), and this further supports the idea that the enzyme directly attacks the embryonic tissue, for it is here that the embryo is most susceptible to the action of unfavourable environmental factors. (Hyman in 1921 exposed *Fundulus* eggs to lethal concentrations of cyanide solutions and found that the tail died first.)

THE EGG CAPSULE.

The chemistry of the salmonid egg shell is not known in detail. Steudel and Osato [1923] and Osato [1923] concluded from the nature of the amino-acids in herring egg shells that the fish egg is homologous only with the yolk of the bird's egg, while the shell of the latter is a new modification for terrestrial life. The albumin of the bird egg corresponds to the mucilaginous covering outside the shell of amphibian and some fish eggs, and the vitelline membrane is the homologue of the fish egg capsule. (The loose use of the word "chorion" for a fish egg shell suggests a homology which does not exist.) It follows that an aquatic egg shell may take a more active part in the scheme of development than a terrestrial one.

After fertilisation the capsule is pale in colour, of a dull sheen, and relatively thick and brittle. It can be stained intensely black with osmic acid [Scheminzky and Gauster, 1924]. Its strength increases for a time, but before hatching it becomes noticeably thinner, somewhat loose and folded, easily torn, and loses its sheen. Its reaction to osmic acid at this time indicates a decrease in fat, or at least of the lipins containing unsaturated acids. Shortly before hatching the water increase in the egg causes the shell to become taut. Many eggs may be hatched at this stage by gently squeezing.

Nordgaard [1896] observed that if a dead opaque salmon egg were placed in a salt solution it became transparent. Gray [1920] found that any of the usual ways of killing a trout egg, such as heat, cold, electric shock, physical shock, etc., were followed by exosmosis of the electrolytes and this caused precipitation of the globulins with resultant opacity. The amount of exosmosis under various conditions could be quantitatively measured from the variations in the electrical resistance of the water in which the eggs were dying. The re-solution of the globulins in normal saline demonstrated that under the unfavourable conditions the capsule had become permeable. The temporal relation between exosmosis and globulin precipitation was determined by Scheminzky and Gauster [1924], who found that the electrolytes leave the egg before the globulins are precipitated. Thus the primary injury is to the membrane rather than to the contents of the egg. Now Stockard [1921] and a number of other workers have demonstrated critical stages in embryonic development, at which it is particularly easy to subject an egg to a shock which will later produce an embryonic deformity. These conditions are generally attributed to changing properties of the protoplasm (varying oxidation rates of different parts at different times, etc.), but the final explanation may well be found in the egg membrane. It is worth noting that salmonids are the fish in which embryonic deformities have been most frequently observed in nature.

Scheminzky and Gauster themselves do not believe that the varying susceptibility during development is due to the membrane, because the electric current required to kill an egg in the later stages increases, while the tensile strength of the shell decreases. (Hein [1907] determined the pressure necessary to crush an egg at various stages.) There is, however, no good reason to believe that permeability is determined by strength and thickness. Furthermore, during development a new membrane system comes to surround the yolk. In later stages two membrane systems, a band of muscle and a fluid-filled coelom, must be penetrated in addition to the shell before the yolk is reached. An inspection of Scheminzky and Gauster's fig. 19 suggests that the coelom surrounds the yolk at the 24th day, since the resistance of the egg rises suddenly at this time.

It has been agreed by most workers that the capsule of the living egg is normally permeated by water but not by electrolytic or colloidal substances. Svetlov [1929] has recently sharply criticised this conception. Using micromethods he found a lower osmotic pressure in the perivitelline fluid of trout eggs than in the yolk, and concluded that the egg contents have the power of maintaining their osmotic pressure independently of the capsule. This does not deny the same function to the cell membrane. Kite [1913] found that most animal and plant cells regulate their permeability as much by the cytoplasm as by the cell wall.

Svetlov also found that salt ions passed through the capsule of a living egg and into the perivitelline fluid. Now Loeb [1912] has shown that the action of a solution of one salt on *Fundulus* eggs is to increase the permeability of the capsule. If a solution of two antagonistic salts such as NaCl and $CaCl_2$ were used there was no changed permeability. What Svetlov has really done therefore is to injure the membrane with an electrolytic solution, the ions of which afterwards passed through. Loeb points out that a dilute acid or alcohol solution has the same effect as a salt solution, and he says, "The increase in the permeability of the membrane for water and salts under the influence of acids is due to an alteration of the protein constituents of the membrane." Thorner [1929] immersed developing *Fundulus* eggs in KCl until the heart ceased to beat, and then removed them to fresh water or sea-water. In distilled water there was a slow extrusion of salt through the shell until after some days the heart recovered. The reaction in sea-water was quite different, the time required for recovery being short and directly proportional to the period of exposure to KCl. Since *Fundulus* eggs can develop in either fresh water or sea-water the customary reaction of the shell must be the same in both media. It follows therefore that after immersion in one salt the capsule behaves abnormally. Sumwalt [1929] found that the electrical potential of *Fundulus* eggs was changed to a marked degree if the eggs were immersed in solutions of various salts. In neutral solutions the capsule was more permeable for cations than for anions. Bivalent cations had about half the effect of univalent cations. As the acidity of the solutions was increased the cation and anion permeabilities approached one another until at p_H 3·7 they were equal. In more acid solutions reversal of permeability occurred. Sumwalt reviews the literature on this point which serves to show the pronounced effect of salt and acid on the capsule. Svetlov mentions a simple and convincing proof of the permeability of the capsule to salt under normal conditions. If a very young egg, which has become opaque while the protoplasm is still alive, is placed in normal saline the opacity disappears and normal development may continue; but as soon as it is replaced to tap water it dies. This really proves that it is possible to compensate for an injured capsule by rearing the egg in normal saline. The capsule, however, never returns to its normal condition as is shown when the egg is replaced to tap water.

Svetlov's vital staining experiments seem at first sight to prove his contentions. If eggs were immersed in neutral red or Nile blue sulphate the dye passed through the capsule and stained the contents intensely. Negative results followed similar experiments with trypan blue and Indian ink. Neutral red required 3 hours for the maximum effect, and Nile blue sulphate 12 hours. It is concluded that the egg capsule is permeable to some colloids under natural conditions. The first thing to notice is that neutral red and Nile blue sulphate belong to a toxic group of stains. Romeis [1928, p. 198] gives particular warning that even in highly dilute solutions neutral red is poisonous. Secondly, teleost embryos have been shown by Wislocki [1917] to be incapable of taking up colloidal dyes in which they are immersed. Their impermeable integument distinguishes them from amphibia larvae [see McClure, 1918].

The writer has carried out vital staining experiments with the salmon (*Salmo salar*), the plaice (*Pleuronectes platessa*), and the goby (*Cyclogaster liparis*). Eggs were immersed in solutions of neutral red, methylene blue, ponceau S., and Janus green. Five hours' immersion in a 0·1 % solution of any of these stains coloured the capsule intensely, methylene blue being most readily adsorbed. When the shell was removed there was absolutely no trace of dye in the egg contents. Similar results followed three days' immersion in the dye. The perivitelline fluid of salmon eggs placed in a saturated solution of methylene blue for 15 hours was slightly stained, but the larvae within were either dead or dying. There was no case in which the stain passed through the shell and the embryo lived. The positive results only showed the staining reaction of dead tissue to dye-stuff.

A number of artificially hatched salmon larvae were left for 24 hours in dilute solutions of neutral red, methylene blue, trypan blue, and nigrosine. On examination they were found to be unstained. Using a 1 % solution the results were the same as long as the larvae lived, but when they were about to die a diffuse staining occurred. With plaice and goby larvae after hatching the results were the same. Thus Wislocki's observations have been entirely confirmed.

It is concluded that in Svetlov's experiments the dye injured the capsule before passing through, and injured the living tissue before staining it. In the absence of further evidence we may continue to believe that under natural conditions there is no exchange of electrolytic and colloidal substances through the shell.

The recent work of Straub [1929] presents the egg capsule in a new rôle. He analysed the causes of observed differences between the osmotic pressures in the yolk and white of hens' eggs. The vitelline membrane is permeable to both water and dissolved substances, nevertheless in the living egg the yolk keeps a higher osmotic pressure than the white for weeks. The difference is lost very quickly in an egg killed by cold. It requires a measurable expenditure of work on the part of the egg to maintain this condition. In general it is in the form of electricity that the free energy of a chemical reaction can be made to do work. The vitelline membrane must be expected to have the properties of a galvanic element, that is, some of the intermediate stages of glucose oxidation must take place in such a way that their free energy maintains a constant difference in potential across it. The membrane bears the same relation to the system as the copper wire between the poles bears to an electric battery. Neither is capable of acting alone as a producer of differences in potential. Straub predicts the histological structure that will be determined for the vitelline membrane of a hen's egg—a porous sheet in which the fluid-filled holes and solid parts provide the liquid and solid phases necessary for the passage of electric currents in both directions.

VARIATIONS IN WEIGHT AND THE RÔLE OF WATER IN DEVELOPMENT.

The data under discussion in this section are summarised in Tables I and II, and in Figs. 1 and 2. The curves are broken at hatching time owing to the loss of the shell and perivitelline fluid. Both egg and larval determinations have been made on the 65th and 70th days. The time over which hatching normally extends is shown on each graph by dotted ordinates, and broken lines give a similar indication of the approximate time during which the effects of hatching are felt. The special metabolic features associated with hatching show with particular clearness in the last eggs to hatch, and the changes in the curves caused by these eggs are of particular interest. As the larvae were selected from the main group at random the special features appear in varying degrees, so that before the effects of hatching are over some irregularity in the determinations occurs. In other words the internal factors—enzymes for example—which adversely affect the eggs during the last stages must injure the late-hatching larvae most.

Table I. *Variations in the dry material and water content during development.*

1 Days after fertilisation	2 Material	3 Wet weight	4 Dry weight	5 Water (%)	1 Days after fertilisation	2 Material	3 Wet weight	4 Dry weight	5 Water (%)
36	*e*	13·2784	4·3568	67·3	76	*l*	11·8804	3·9194	67·0
40	*e*	13·1162	4·2826	67·2	79	*l*	11·5903	3·7928	67·3
44	*e*	13·2764	4·2956	67·6	86	*l*	11·8374	3·7154	68·6
46	*s*	0·6750	0·2065	69·5	90	*l*	11·4210	3·4992	69·4
50	*e*	13·3762	4·3110	67·8	94	*l*	11·2256	3·4052	69·7
55	*e*	13·2046	4·3026	67·5	100	*l*	11·2000	3·2372	71·1
60	*e*	13·4595	4·3122	67·9	106	*l*	11·1556	3·0408	72·7
65	*e*	13·3143	4·2740	67·9	111	*l*	11·1808	2·9931	73·2
65	*l*	11·2842	4·0254	64·5	116	*l*	11·0876	2·8720	74·1
70	*e*	13·9796	4·2802	69·5	124	*l*	10·8034	2·5308	76·5
70	*l*	11·6766	3·8832	66·8					

Absolute figures represent the weights for 100 individuals in grams. In col. 2 *e* means eggs; *s*, shells; *l*, larvae.

Table II. *Growth of embryos and plastic efficiency coefficients.*

1 Days after fertilisation	2 Embryo wet weight	3 Yolk wet weight	4 Embryo dry weight	5 Yolk dry weight	6 Plastic efficiency coefficient days	 coeff.
36	0·223	12·450	0·036	4·115	—	—
86	1·113	10·724	0·175	3·543	—	—
90	1·612	9·809	0·261	3·235	86–90	0·413
94	1·784	9·442	0·288	3·116	90–94	0·094
100	2·698	8·502	0·432	2·804	94–100	0·462
106	3·744	7·414	0·598	2·445	100–106	0·462
111	4·076	7·105	0·652	2·342	106–111	0·524
116	4·630	6·458	0·741	2·132	111–116	0·424
124	6·035	4·768	0·962	1·573	116–124	0·396
—	—	—	—	—	36–124	0·364

Fig. 1 shows that there is no measured decrease in the dry weight until a few days before hatching. This is in line with the idea that there is no loss of substance through the shell. Just before hatching the membrane becomes thin and loose, permitting an increased water content. The previously noted loss of fat in the egg capsule during development may partly account for the decrease in dry weight. In the last stages the capsule has become permeable and substance has passed through. Very little movement is possible for the larva within the egg, but after hatching it can swim about freely. The increased activity causes a more rapid loss in dry weight until an unexpected plateau appears at the 106th day, the former slope being resumed again 10 days later. May it not be that this period is one in which there is a change in the nature of the sources of energy with which the yolk provides the embryo for its building operations?

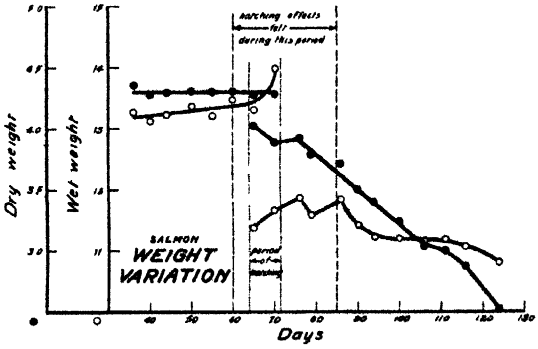

Fig. 1.

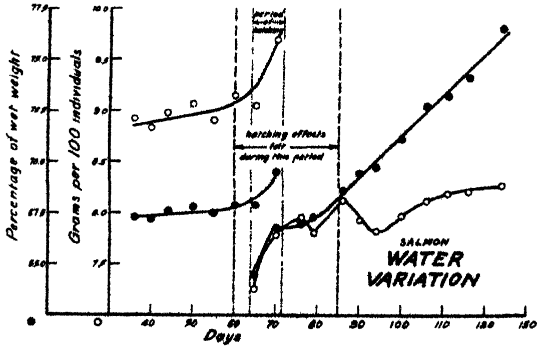

Fig. 2.

Studies on the mechanics of development of trout eggs and larvae have recently come from two sources. Since they differ somewhat with respect to observations as well as to theoretical conceptions it is necessary to examine them here with a view to determining which agrees more closely with related facts.

Kronfeld and Scheminzky [1926] measured the embryonic length and the wet and dry weights of the embryo, yolk sac, and egg capsule during development. The species used were *Trutta fario* and *Salmo lacustris* as well as hybrid eggs. In each experimental series they used eggs of one group with unfertilised eggs as controls. The method involved preliminary hardening in 2 to 4 % formalin, but they produce evidence to show that this caused no significant changes. The water content of the egg as a whole did not appreciably vary up to the time of hatching. They have not distinguished between early and late hatching eggs and have therefore missed the sudden rise in the percentage of water which occurs just before the hatching of the latter. There was a loss of water at hatching, owing to the loss of the capsule and perivitelline fluid, followed by a rapid rise which continued until the end of development. They found that the water content of the embryo alone dropped until hatching and then rose suddenly, to reach its final level of 86 % three weeks later. The yolk sac alone contained 66 % of water in the early stages. From this tension the water dropped to as low as 50 % a week after hatching, followed by a rise so that the normal figure was reached again two weeks later. The developing embryo faced the problem of building living tissue of high water content from yolk of low water content. Presumably the living cells could only absorb water through the yolk sac, and for some days after hatching the water taken was not replaced. When the living tissue was almost in equilibrium again the water in the yolk sac commenced to rise. There was evidently some delicate adjustment of organism to environment, on the nature of which we may be able to speculate after considering the chemical changes [Hayes, 1930].

An investigation was also made of the specific growth rate during development, that is how much new tissue can be built per unit embryo per unit time at different ages. According to Kronfeld and Scheminzky's fig. 4 the curve of building power rose to two peaks, one of them 16 days before hatching, and the other 23 days after hatching. Anatomically the first maximum corresponded to the formation and growth of the chief organs of the body, the ensuing drop being due to a form of starvation owing to lack of water. The drop after the second maximum they attributed to the lack of some necessary material in the yolk, and they point out that this is the time at which hatchery experience has shown it is best to begin feeding the larvae.

Similar investigations to those just described have been carried out on the same species of trout (*Salmo fario* in British terminology) by Gray [1926, 1928]. The variations of the wet and dry weight of the egg or larva, embryo, and yolk (by difference) were found. The figures presented show that the embryonic water content remains constant at approximately 84 %, and that of the yolk at 59 %. These results indicate that the hatching process does not affect the water content of the yolk or embryonic body. The dry weights given in the second paper are calculated on this assumption.

Gray has also given figures for the specific growth rate during development, which are in agreement with those of Kronfeld and Scheminzky. His observations began at the 44th day, or after the time of the first maximum. The two curves inflect when the larva has completed about 70 % of its development. The agreement on this point may be accepted as proof that by this stage both the embryo and yolk have an unchanging water content. It is possible to calculate the weight of the salmon embryo and yolk at this stage by weighing the whole larva wet and dry (see below). According to all workers the larval wet weight increases for some time after hatching, and then decreases until the embryo begins to take outside food. The water content however (Fig. 2), after the fluctuations subsequent to hatching, rises steadily until the embryo begins to feed. Therefore in the later stages the loss in dry material is greater than the gain in water. Increases in water are associated with the transformation of yolk into living tissue, and losses in dry substance are due to the catabolism of the embryo.

In Table II the plastic efficiency coefficients represent the ratio

$$\frac{\text{gain in embryonic dry material}}{\text{loss in yolk dry material}}.$$

This coefficient has been determined for a number of forms, and in a given species it may be assumed to be of the same order throughout larval development. It is given here for the salmon with the object of showing that by determinations of the wet and dry weight of the larva it can be decided independently whether the embryo and yolk have a constant water content. Data from the egg are invalid since two unknown variables are included, the shell and the perivitelline fluid. Assume, as Gray did, that both embryo and yolk actually maintain a stablised water content which may be found experimentally and applied to larvae of other ages. The dry weight of the embryo, yolk or larva is equal to the wet weight multiplied by the percentage of dry substance. The total larva is equal to the sum of the embryo and yolk. Instead of "yolk wet weight" can be written "larva wet weight minus embryo wet weight." Hence the following equation is true:

$$\%\text{ dry wt. larva} \times \text{wet wt. larva} = \%\text{ dry wt. yolk (wet wt. lar.} - \text{wet wt. emb.)} + \%\text{ dry wt. emb.} \times \text{wet wt. emb.}$$

The only unknown is the embryonic wet weight which is found by solving the equation. The equation can be conveniently expressed by a formula. Let T = total wet weight of larva given in col. 3, Table I; let x = total wet weight of embryo; let a = % dry weight of larva found by subtracting the value in col. 5, Table I from 100; let b = % dry weight of yolk experimentally found to be 33 in later larval stages; let c = % dry weight of embryo experimentally found to be 16 in later larval stages. The value 16 for c is among the highest found, but the position of any possible inaccuracy will be obvious when one remembers that it is dependent on the removal of all moisture from the outside of very small embryos dissected away from their yolk sacs. The fresh embryos used were fragile and it was difficult to remove all the water. The use of the figure 16 brings the theoretical results into harmony with direct observations. The following is the equation:

$$aT = b\,(T - x) + cx,$$

or it may be written

$$x = \frac{(b-a)\,T}{b-c}.$$

An attempt was made to apply this formula to each of the larval weights determined. The 65th and 70th day calculations gave the embryonic weight as a minus quantity; the 76th day as exactly zero; and on the 79th day 1·715 g. or double the experimentally observed value. The figures from the 86th day forward are given in col. 2, Table II, and in col. 6 the plastic efficiency coefficient (P.E.C.) is recorded. The latter figures (with one exception due to experimental error) agree quite well and show that after the 86th day the embryo and yolk have a constant proportion of water. The lack of uniformity of the calculations made before the 8th day provides independent evidence in favour of Kronfeld and Scheminzky's statement that the water content does not become stabilised for some time after hatching.

Col. 6, Table II also compares the P.E.C. over a long period (36 to 124 days) with that of the later larval stages. From this it may be seen that the coefficient increases somewhat during development. The 36-day figures quoted were obtained in the usual way and a correction applied for the shell. Their accuracy is probably not of a very high order, but this is offset by the small size of the embryo.

The form of the growth curves given by Gray, and Kronfeld and Scheminzky shows that after the second maximum an ever-increasing proportion of the yolk supplied is burned to provide energy of maintenance, while a decreasing share is built into new tissue. In other words basal metabolism steadily replaces growth. If no outside food were provided the embryo would finally cease to grow, become emaciated, and die. Thus if the P.E.C. given above had extended until the disappearance of the yolk sac it would necessarily have declined, indicating that the larva was wasting more food at the end of development. The conceptions of growth recently developed by Terroine and his collaborators show that this is not really the case. [Complete references may be found in Terroine and Wurmser, 1922, and in de Caro, 1928.] We are dealing here only with matter, and leaving out of consideration the energy of maintenance of the cells already formed. Any cell must carry on metabolism if it is to remain alive and this basal metabolism is not waste. The real energetic efficiency must be calculated according to the formula

$$\frac{\text{energy gained by embryo}}{\text{energy lost by yolk} - \text{energy of maintenance}}.$$

Some observers have attempted to distinguish between non-basal metabolism (wasteful burning of food) and basal metabolism (energy of maintenance) by measuring the surface area and assuming that the latter bears a constant relation to basal metabolism. Not wishing to rely on surface laws Terroine and Wurmser made their experiments directly, by finding the way in which the growth of slime moulds was hastened or retarded by adjustments of the p_H of the culture medium. The real developmental efficiency of different embryos, such as the frog, silkworm and chick, is of the same order. They waste from 20 to 30 % of the energy provided for their development. Accurate figures for the real energetic efficiency of a fish are not yet available.

SUMMARY.

1. The wet weight of a salmon egg increases slowly until a short time before hatching, then rapidly. In the larva the wet weight first increases and then decreases. The dry weight drops rapidly after hatching.

2. Observations on hatching show that the enzyme which dissolves the shell has a very unfavourable effect on the embryo.

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(*Note added to proof.*) In a further contribution (*Pflüger's Arch.* 1929, **223**, 645), Scheminzky discusses at length the discrepancies between his results and those of Gray. If Scheminzky discards his own experimentally obtained figures for the variations in embryonic water content during development, and assumes as Gray does that this is constant, he is able to apply Gray's equations to his other results and obtain a similar curve. He finds an agreement in experimental observation, the disharmony of the conclusions being due, first to the above mentioned assumption, and second to a difference in mathematical treatment. "Die Wachstumskurve nach *Gray* stellt somit einfach die in einem bestimmten Zeitpunkt vorhandene Menge von embryonaler Substanz dar, *ohne über die Intensität* des Wachstums eine Aussage zu machen."

LXXXIII. THE METABOLISM OF DEVELOPING SALMON EGGS.

II. CHEMICAL CHANGES DURING DEVELOPMENT.

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INTRODUCTION.

THERE is much evidence in the literature of embryonic metabolism which goes to show that during the course of its development the embryo makes use of foodstuffs as energy sources in the order carbohydrates, protein, fat. With this idea in mind Needham and Murray [see Needham, 1927] have recently ascertained the wet and dry weights, and the constitution, concentration, combustion, and absorption of yolk materials, of the developing embryo. The peak of carbohydrate combustion and absorption was found to be on or before the 4th day. With protein there was no correlation whatever between absorption and combustion; the peak of combustion at the 9th day came just at the trough of absorption, and by the time the peak of absorption was reached on the 16th day combustion had ceased to be a factor. A similar statement can be made for fat, the absorption of which rose to a peak by the 9th day, dropped to a trough by the 15th day and then rose until the end of development. Thus fat and protein complement each other during the later stages as sources of material for the formation of living tissue; their curves are reciprocals. It is probable that the cells of the blastodermal blood vessels which absorb the yolk and white into the embryo, have periods of varying permeability to water-soluble and fat-soluble substances. Of the end-products of protein combustion ammonia reaches a peak first, then urea, and finally uric acid. At the time of transference from protein to fat as a chief source of energy the cost of constructing 100 mg. of living tissue is greatest. The results of these workers are mentioned because they suggest the explanation to so many of the observations here recorded. It cannot be stated at present exactly to what extent the fish resembles the bird in the chemistry of its development. The scarcity of carbohydrate in fish eggs accounts for one difference.

Chemical analyses of Atlantic salmon (*Salmo salar*) eggs are not available but Greene [1921, 1, 2] removed mature ova from the body cavity of the king salmon (*Oncorhynchus tschawytscha*) and determined the following percentage composition: water 57·93, protein (alcohol precipitate) 26·79, neutral fat 10·42, phospholipins 2·0, organic extractives 1·42, glucose 0·09, inorganic ash

0·65. For trout eggs (*Trutta fario*) Fauré-Fremiet and Garrault [1922, 1, 2] have found the following percentage composition: water 58·5, protein (total nitrogen × 6·25) 29·81, neutral fats 4·20, phospholipins 3·46, cholesterol 0·58, carbohydrates 0·34, ash 1·25. They were unable to find glycogen in the egg, but determined that the reducing sugar isolated was glucose.

Some recent work, which will be further discussed, has led to the conception that an aquatic egg is essentially different from a terrestrial one in two ways. First the products of its protein combustion are excreted through the shell, and second the supply of water for the developing embryo is unlimited. The latter idea has been discussed at length in the foregoing paper [Hayes, 1930]. Protein metabolism is held to be limited in terrestrial eggs by some factor connected with the increasing concentration of nitrogenous end-products. Thus protein is a minor source of energy in terrestrial embryonic development, and a major source in aquatic embryos. Another explanation is possible, using the ever attractive theory of recapitulation. Up to about the 7th day the chick embryo develops like a fish. It combusts and absorbs chiefly protein. Then the vitelline membrane (corresponding to the fish egg shell) finally disappears and its place is taken by the serosa. The embryo gradually loses its dependence on protein, ceases to be a fish, changes into a bird. The fish at this stage hatches and continues to rely on protein as the main source of food supply during larval development.

MATERIAL AND METHODS.

Eggs of the Atlantic salmon (*Salmo salar*) were procured in the eyed stage, reared, weighed, and dried as described in the first paper of this series. The dried material was then analysed for protein and fat.

Fat was extracted in a Soxhlet apparatus, the eggs having been previously pulverised; this latter procedure was unnecessary with the larvae. Extraction was allowed to proceed in the usual way for 3 hours with the siphon operating every 5 minutes, re-extraction of some of the samples having shown that this period was of sufficient duration to remove all the fatty constituents. After extraction the solvent was distilled off and the residue and extract dried to a constant weight. The extract was always slightly heavier than the residue loss. The former has provided the data recorded, except in one or two cases of obvious experimental error in which the residue loss, appropriately corrected, has been used.

The fat values given are the result of carbon tetrachloride extraction. The relative merits of ether and carbon tetrachloride as fat extractives have been discussed by several workers. Johnstone [1918] found 1·0 to 1·62 % of nitrogenous substances in carbon tetrachloride extracts of herring tissue. Bull [1928] preferred to use ether for similar work. It is always desirable, and particularly so in studies of developmental mechanics, to differentiate between lower nitrogenous substances such as protein building stones, aminolipins, etc., and the true proteins. Greene [1921, 1] calls the former substances "organic extractives" to distinguish them from the neutral fats and phospholipins. In more general analyses they should be placed with the fats rather than with the proteins. Carbon tetrachloride is to be preferred as an extractive if it will remove these lower substances and thus permit a more accurate estimate of the proteins in the residue. If its extract contains slightly more nitrogen than ether extract of similar tissue it is fair to assume that this excess represents the simplest organic nitrogenous substances present. Differences in extractive power may be entirely due to temperature. In ordinary procedure ether extracts at slightly below its boiling point of 35°, while carbon tetrachloride extracts at slightly below 77°. Greene considered it necessary to modify his apparatus in order to ensure the highest possible temperature for extraction.

The first point was to determine whether there was a quantitative difference between the two types of extracts, and this was done using eggs of the common lumpsucker (*Cyclopterus lumpus*). Three samples were extracted with ether, then dried, weighed and extracted with carbon tetrachloride. The averaged results showed that 98·5 % of the total extract was removed by the ether and the remaining 1·5 % by the carbon tetrachloride. Estimations showed that the percentage dry weight of nitrogen in ether extracts is 0·287, and in carbon tetrachloride extracts it is 0·354. Combining these results we find that 19 % of the total nitrogen is contained in the carbon tetrachloride fraction. In other words the 1·5 % extra fat which the carbon tetrachloride removes contains 6·73 % nitrogen, a figure which is not far from the proportion in the egg as a whole. This extra nitrogen, however, is not capriciously taken from the main store of protein. Longer or repeated extraction will never remove any more, so that it must represent a specific type of compound in the egg—some product of protein breakdown. In a developing system such rearrangements are of the greatest interest, so that carbon tetrachloride, which differentiates to a greater extent than ether, is to be preferred as an extractive. It must be further noted that the nitrogen removed is in any case a relatively small fraction, amounting with carbon tetrachloride to 0·924 and with ether to 0·707 % of the total in the egg. Thus in about 25 % of the dried tissue extracted as fat considerably less than 1 % of the nitrogen is included. All these figures have been checked by another method, namely the analysis of eggs for nitrogen before and after extraction. The two sets of results showed close agreement.

Nitrogen was estimated by the Kjeldahl method, using 0·5 g. of dry fat-free substance. For percentage values it was necessary to apply a correction for the extracted fat. After some consideration it was decided to follow the classic practice of stating that the protein factor 6·25 is incorrect for fish tissues, pointing out why it is incorrect, and then using it. The question of non-protein nitrogen is discussed elsewhere.

The ash was determined by gently heating 0·5 g. of dry fat-free substance in a lightly covered silica crucible for 1 hour, then applying intense heat until the ash became colourless. Bruce [1924] has pointed out that some of the volatile chlorides are driven off during the heating and some phosphorus in organic combination may be extracted with the fat, but these small quantities are within the limits of experimental error.

The Winkler method was used to determine the oxygen requirements of the developing eggs and larvae. A large receptacle was filled with tap water and allowed to stand until equilibrium with the atmosphere had been reached. Water was then siphoned from the receptacle into a 6-litre jar of accurately known capacity, from which samples were taken for oxygen estimation. One group of 1000 eggs was used for all the tests, these being placed in the jar and the lid sealed on, avoiding included air. Sometimes a bubble appeared in the jar during the course of the experiment, probably released from the slightly supersaturated water, but the error so introduced was not great enough seriously to affect the results. The duration of each test with eggs was 2 hours, a period in which less than 10 % of the available oxygen was used. The suddenly increased oxygen requirements after hatching necessitated a change in the routine, and 200 larvae were allowed to breathe for one hour in the jar. Burfield [1928] showed that the oxygen consumption rate of plaice eggs decreases in a closed vessel owing to the small quantities of carbon dioxide produced. Respiration was normal with a greatly diminished oxygen supply. The analyses here given indicate that the respiratory quotient is almost constant throughout development, so that by the standard methods adopted any decrease in oxygen consumption is the same in all cases and can be disregarded. During experiments the large jar was kept immersed in a tank to ensure standard temperature conditions. The eggs or larvae were kept frequently in motion by turning over the jar every 15 minutes. At the conclusion the lid was removed and water samples taken. A siphon reached to the bottom of the sampling bottle and water was allowed to flow over the top until three times its volume had passed through. Then the siphon was removed and the bottle stoppered without included air, so that the sample taken had not progressed toward equilibrium with the atmosphere. All determinations were made in duplicate, and care was taken frequently to standardise the sodium thiosulphate solution against potassium permanganate solution, which was itself frequently standardised against potassium tetra-oxalate. Infrequently one of the readings was wide of the mark and was discarded; in other cases the figures quoted are averaged from all four determinations.

PROTEIN VARIATIONS DURING DEVELOPMENT.

The results of the protein determinations are shown in col. 3, Table I, and in Fig. 1. The first important point is that the larva has a larger proportion of protein than the egg. The sudden rise at hatching can only mean that the protein is relatively more concentrated in the larva than it is in the shell and perivitelline fluid. But an analysis of egg capsules three weeks before hatching shows that the protein and water content are higher than in the egg as a whole. Some of the protein in the egg capsule has been destroyed before hatching by the secreted enzyme. The rise in the total protein of the egg just before hatching is due to the method of calculation, which assumes that all the nitrogen is protein-nitrogen, which is never quite true and sometimes not as approximately true as at other times. Nitrogen is present in forms which are extracted with the fat (see next section). The measured protein content of the larva rises because yolk-protein of low nitrogen content is transformed into living tissue-protein of high nitrogen content. The sudden percentage decrease before hatching is merely a reflection of the greatly increased water content.

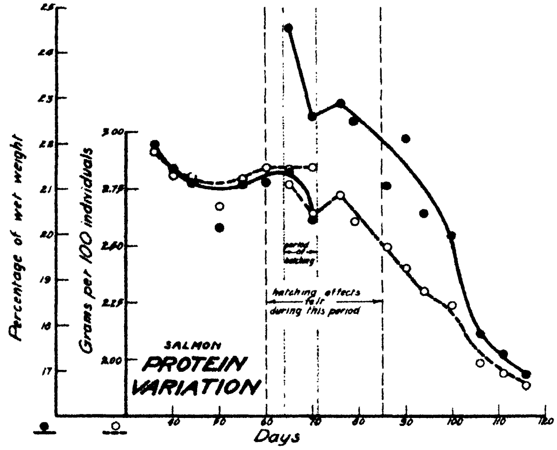

Fig. 1.

Table I. *Variations in the chemical composition and oxygen consumption during development.*

1 Days after fertilisation	2 Material	3 Protein %	4 Fat %	5 Remainder %	6 Smoothed remainder %	7 Oxygen consumption
36	*e*	21·98	3·98	5·49	5·45	0·060
40	*e*	21·42	4·94	5·19	5·31	0·075
44	*e*	21·17	4·81	5·17	5·17	0·068
46	*s*	22·30	—	—	—	—
50	*e*	20·15	4·93	5·87	4·97	0·066
55	*e*	21·20	5·01	3·04	4·80	0·055
60	*e*	21·15	5·00	4·70	4·63	0·052
65	*e*	21·43	4·94	4·48	4·46	0·049
65	*l*	24·54	3·00	6·71	5·49	—
70	*e*	20·35	4·47	4·63	4·29	—
70	*l*	22·60	4·20	5·15	5·16	0·433
76	*l*	22·90	4·06	4·79	4·77	—
79	*l*	22·50	4·06	4·89	4·58	0·471
86	*l*	21·05	5·83	3·17	4·12	—
90	*l*	22·10	5·63	1·62	3·86	0·729
94	*l*	20·45	4·87	3·73	3·59	0·629
100	*l*	19·96	5·16	2·53	3·20	0·956
106	*l*	17·80	5·41	2·94	2·82	—
111	*l*	17·36	5·06	3·13	2·49	—
116	*l*	16·91	5·18	2·54	2·18	—

Each oxygen figure is the requirement of 1000 individuals in 2 hours. Absolute figures represent the weights for 100 individuals in grams. For "remainder" calculations before hatching the averaged ash value of 0·16 g. or 1·25 % is used; after hatching 0·134 g. or 1·25 %. In col. 2 *e* means eggs; *s*, shells; *l*, larvae.

The activity of the free-swimming larva is shown by the rapid rate at which the protein is burned after hatching. The suddenly increased rate of combustion at the 100th day followed by a diminution 10 days later, marks this as a critical period in development. Morphological considerations offer no solution. Now Needham [1927] found that in the chick the point of maximum protein combustion is also the point at which the greatest quantity of substance is burned per unit of new tissue formed (this is not necessarily true on theoretical grounds), and it is just at this period that the transfer from fat to protein is going on. He cites Terroine, Trautman and Bonnet's results showing that such a transference results in an extra energy loss of 23 %. In the salmon fat begins to disappear at this point, and it also corresponds to the second growth rate maximum in the trout. A morphological examination shows that there is still an abundance of protein in the yolk sac. It is the physico-chemical properties of the organism and not a lack of food which determine why it should suddenly start to dilute its protein diet with fat.

The "remainder" values to be found in Table I and in Fig. 2, have been found by subtracting the total of the other values from the total weight of the egg or larva or from 100 (col. 5), and then smoothing these values into two straight lines corresponding to the periods before and after hatching (col. 6). (The values for total weight and water content are given by Hayes [1930], Table I.) The smoothed values were calculated using the simplest case of the method of moments where

$$y = a + bx$$

$$a = \frac{1}{2l} m_0$$

$$b = \frac{3}{l} \cdot \frac{1}{2l} \cdot \frac{m_1}{l}$$

y = vertical ordinate; x = distance from centre of range; $2l$ = range; m_0 = total area (sum of the frequencies); and m_1 = first moments about the centre of the range. The method is described by Elderton [1906]. The curves in Fig. 2 have been plotted along the smoothed values, although the points shown represent only the unsmoothed figures. (The ash content showed no significant variations during development. Its average for 100 eggs was 0·160 g.; for 100 larvae 0·134 g. The percentage throughout development was 1·25. These constants have been used to calculate the "remainder.")

Since the "remainder" values involve so many sources of error a high degree of conformity between the smoothed and unsmoothed values cannot be expected, but the points show clearly that at the time of hatching the slope of the curve suddenly steepens. The possible carbohydrate content of the eggs is small and cannot explain the "remainder." Water, fat and ash contents have been determined directly and any possible error could not vary with the developmental stage. With the protein, however, the procedure of multiplying the total nitrogen by 6·25 (or any other factor) involves the assumption that the relation of nitrogen to protein is the same in different stages of development and in different egg constituents. From the entrance of the spermatozoon until the larva begins to take outside food there is nothing added to the egg except water, so that all the nitrogen must have been there from the beginning although more of it was missed in the earlier stages than later. Levene [1901] has shown that the nucleoproteins of the cod egg, which are especially rich in nitrogen, and moreover are required for the formation of cell nuclei, increase during development. The period of salmon development considered is one of rapid growth, and differences in constitution between embryo and yolk show with increasing clearness in successive analyses. Probably increasing nucleoprotein explains the form of the "remainder" curves. Stated otherwise the curves show that in the system as a whole the amount of protein corresponding to one unit of nitrogen is greater in the earlier stages than later, while the method of analysis assumes that it does not vary.

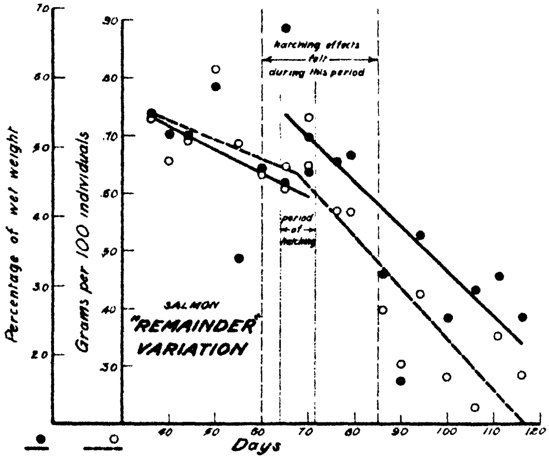

Fig. 2.

Since this difference between yolk and embryonic protein is a true one it is interesting to gain some idea of its magnitude. By what figure should the total nitrogen (after fat extraction) be multiplied in order to find the total embryonic or yolk protein? It is necessary to assume that substances other than water, protein, fat and ash are present in such small quantities that they may be disregarded. In col. 5, Table I, there is a "remainder" of 5·49 for the 36-day eggs. This means that on the above assumptions the correct protein value should be 27·47 instead of the 21·98 shown in col. 3. To obtain this value a factor of 7·81 must be used. From cols. 2 and 3, Table II [Hayes, 1930], the weight of the embryo and yolk at this age may be obtained and expressed as percentages; they are 1·76 and 98·24 respectively. If the same data are sought for the 116-day larva it is found that the larval protein factor necessary is 7·19, the embryonic weight 41·8, and the yolk weight 58·2. Let $x =$ the embryonic protein factor, and let $y =$ the yolk factor, and the following equations are true:

$$\begin{aligned} 1{\cdot}76x + 98{\cdot}24y &= 100 \times 7{\cdot}81 \\ 41{\cdot}8x + 58{\cdot}2y &= 100 \times 7{\cdot}19. \end{aligned}$$

The solution gives x a value of 6·36 and y a value of 7·82, which means that the embryonic protein contains 15·7 % nitrogen and the yolk-protein 12·8 %. Embryonic and yolk weights within the egg and other values could be obtained by the use of these factors, but such calculations are not justified without confirmatory evidence. Research in the choice of a factor in such investigations as this is obviously most desirable.

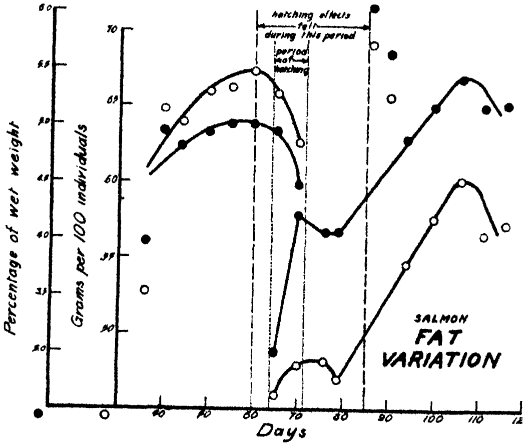

Fig. 3.

Fat variations during development.

The fat extraction results are shown in col. 4, Table I, and in Fig. 3. The absolute and percentage values rise steadily until a few days before hatching, then decline suddenly. Hatching produces a further drop due to the loss of the shell and perivitelline fluid. During larval development the fat maximum is reached at the 106th day, following which both percentage and absolute values decrease. There is every indication that fat is being formed from protein during the early stages and again during the greater part of larval development. Since many fish eggs have very small quantities of fat their ability to synthesise it is a matter of very great importance in larval nutrition. A fat increase during development has been found in trout eggs by Tangl and Farkas [1904] and Gortner [1913], and in plaice eggs by Dakin and Dakin [1925]. With the plaice the amount of fat involved is exceedingly small, amounting to only 0·085 % in the beginning and 0·307 % at the end. Fig. 3 shows that there is a large decrease in late hatching eggs, owing to the necessity for the embryo to burn fat under the special conditions of hatching. Taken in relation to the protein the form of the fat curve suggests that there is one succession of energy sources in the egg and another in the larva.

OXYGEN CONSUMPTION DURING DEVELOPMENT.

The oxygen consumption records are given in col. 7, Table I, and the decrease in hatching is illustrated in Fig. 2. The figures serve plainly to emphasize what appears from all the other experiments; first that hatching produces a complete change in the conditions of development; and second that life processes drop to a low ebb under the unfavourable conditions previous to hatching. The great activity of the free-swimming larva causes its oxygen requirements to be about 10 times as great as in the egg stage.

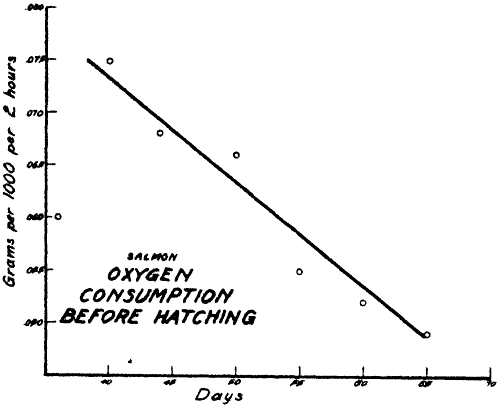

Fig. 4.

Kawajiri [1925] used the Winkler method to investigate the oxygen requirements of the Japanese land-locked salmon (*Oncorhynchus masou*). The variations in the egg stage were negligible, the curve showing a slight trough between the 5th and 10th days. A newly hatched larva consumed 5 times as much oxygen as an egg and the rate of absorption rose rapidly until the end of development. *Fundulus* eggs have also been studied by Scott and Kellicott [1916] and by Hyman [1921]. Their results agree except on one point, the decrease before hatching. Scott and Kellicott failed to find a decrease, but Hyman contradicts this result. Both found that at the time of gastrulation there is a marked rise while during the formation of the embryo there is no further increase. Another sudden rise when the heart begins to beat is followed, according to Hyman, by a gradual decrease until hatching. In the larval stages the oxygen requirements are greatly increased. Both workers followed the same method, namely the estimation of carbon dioxide by the p_H changes of the water in which the eggs were breathing.

THE NATURE OF GROWTH.

In the later stages of egg development there is a gradual increase in water. It is important to know to what extent salmonid eggs are completely closed systems with respect to water, but there is some conflict in the data. In the salmon according to Rünnstrom [1920] and in the trout according to Svetlov [1929] there is a decrease in osmotic pressure during development, showing that water is taken up. Gray [1920] found that in the trout the osmotic pressure of the yolk remains constant during development. Kronfeld and Scheminzky [1926] follow the conceptions of Tangl in regarding the egg as a completely closed system. Since the results given here agree with those of some other workers and have been obtained by direct measurement they may be accepted provisionally.

It had been believed that different osmotic pressures existed in the egg contents, but Svetlov [1929] found (on adequate theoretical grounds) that the embryonic osmotic pressure is the same as that of the yolk. Assuming that substances necessary for growth do not enter the egg from the environment, facts are now at hand for a revised story of the process of embryo building.

As the embryo grows the proportion of salt remains equal to that of the yolk, but that of the organic material is much lower. For building operations there must be a preferential selection of salt. For combustion on the other hand there is an exclusive selection of organic material. Within the egg little movement is possible and combustion amounts approximately to the minimum requirements for basal metabolism. It follows that if the water content of the yolk remained constant its proportion of salt would necessarily decrease. By some property, at the nature of which we can only guess, it maintains a constant salt content by expelling water into the perivitelline fluid which (as Svetlov showed) steadily increases during development. The decline after the first growth rate maximum shows that the increased concentration of colloidal material has changed the properties of the yolk. It cannot become indefinitely more concentrated—there is a plateau in the curve showing yolk water losses (Kronfeld and Scheminzky's fig. 2). In subsequent development the embryo becomes drier and takes more nearly equal proportions of salt and organic material. It also begins to burn fat. Speaking of the chick Needham [1927] states, "Out of 100 g. of protein in its diet the embryo can store away 98; out of 100 g. of carbohydrate 82; but out of 100 g. of fat only 43." If this relationship were true in the fish as well it would offer an explanation of the results. The embryo must maintain its level of water as constant as possible, and the mechanism adopted would be to use an energy source of which a large proportion was burned rather than built into new tissue. At the same time the enzyme which dissolves the shell is secreted and its unfavourable effects appear. At hatching the embryo is suffering from malnutrition, both embryo and yolk are lacking in water, but both have maintained their salt content. In the larval condition the embryo immediately begins to recover. It takes up water rapidly, and salt in proportion, so that the yolk loses water for a short time. The ultimate balance is restored by the greatly increased activity in the free-swimming state until finally the yolk has returned to its early water content level. This point has been shown to correspond to the second growth rate maximum in the trout (Kronfeld and Scheminzky). In the salmon the stabilisation of the water content comes just before the second change to fat as a source of energy. The embryo has grown so rapidly that the conditions of the later egg stages have repeated themselves and the reaction is the same—the selection of fat as building material. There is a decrease in the growth rate and the larva first begins at this time to seek outside food.

These considerations bring the question of the succession of energy sources one step nearer to its solution. Attention is focussed on the salt content, and a fundamental question is to discover why it must remain constant and what happens when it is experimentally changed.

SUMMARY.

1. In the developing salmon the protein decreases in the earlier egg stages and increases before hatching; in the larval stage it is burned rapidly. It has been calculated that embryonic protein contains 15·7 and yolk protein 12·8 % of nitrogen.

2. The fat content rises to two peaks, the first 10 days before hatching, and the second 6 weeks after. The decreases after the maxima are believed to show a change from protein to fat as a source of energy.

3. Variations in oxygen consumption indicate that metabolic processes drop to a low ebb before hatching and greatly increase in the free-swimming larva.

I am very grateful for the inestimable help of Prof. James Johnstone and Dr R. J. Daniel under whose direction this research has been carried out. Prof. J. M'Lean Thompson kindly permitted me to improvise hatching apparatus under especially favourable conditions in the Department of Botany, University of Liverpool. Much of the material used was provided through the courtesy of Mr J. G. D. Watts, Superintendent for the Dee Fishery Board. I also wish to thank the Nova Scotia I.O.D.E. and the International Education Board for financial assistance.

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LXXXIV. THE MICRO-DETERMINATION OF HYDROXYL GROUPS.

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INTRODUCTION.

During the course of other work, it was found necessary to estimate the hydroxyl groups in a substance believed to be a dihydroxy-alcohol [Marrian, 1929], of which only a small amount was available. A search of the literature failed to reveal any suitable micro-method. Experiments carried out on the hydrolysis of acetates of known purity led us to conclude that methods based on this were entirely unreliable when carried out on small quantities. The method based on the volumetric determination of the methane evolved when an alcohol reacts with Grignard's reagent as used by Tschugaeff [1902], Hibbert and Sudborough [1904] and Zerewitinoff [1907] seemed to be the most suitable for adaptation to a micro-scale.

Preliminary experiments made it quite clear that, unless certain precautions were most rigidly observed, entirely irregular results were obtained.

Hibbert and Sudborough emphasise the necessity of working in an atmosphere of nitrogen, since oxygen is absorbed by Grignard's reagent. Zerewitinoff, on the other hand, states that, since oxygen is only slowly absorbed by the reagent, it is unnecessary to work in an atmosphere of nitrogen. In our first experiments, when we did not observe the precaution of filling the apparatus with nitrogen, we were troubled with a rapid and prolonged contraction in volume of the gas in the burette, making it quite impossible to obtain a steady initial reading.

After blowing out the apparatus with dry nitrogen, this contraction was decreased greatly in magnitude, making it possible to obtain a steady initial reading after the lapse of some hours.

Very small variations in temperature and pressure should result in only a very slight error in macro-determinations when the volume of the gas burette is large relative to the total gas volume of the apparatus. In our micro-apparatus, the total gas volume was relatively enormous compared with the changes in volume which were being measured. Consequently small changes in temperature and pressure were observed to introduce appreciable errors. By suitable means the temperature was kept sufficiently constant, but variations in barometric pressure have constantly caused trouble. In unsettled weather, the pressure may change 3–4 mm. during the course of half an hour, making it impossible to obtain steady readings. We found it necessary to observe the height of the barometer constantly during the course of an experiment, and if necessary to abandon the experiment for the day. The only alternative would be to reduce the total gas volume of the apparatus considerably. This would necessitate working with a much smaller reaction flask, which in many ways would be inconvenient.

The usual method of carrying out macro-determinations is to dissolve the alcohol which is being examined in some inert solvent, preferably the ether with which the Grignard reagent is made, and to allow this solution to react with the reagent. The difficulty of drying and keeping dry such a solvent suggested to us that for micro-determinations it would be preferable to allow the reagent to react with the solid alcohol. This method has been found to work excellently provided the alcohol is readily soluble in the ether of the Grignard reagent.

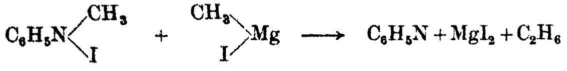
Preliminary determinations showed us that high results were invariably obtained unless the reaction flask and the substance being examined were dried with extreme care. Even so, blank determinations have always given an appreciable volume of gas. The magnitude of the blank seems to vary with the exact technique of cleaning and drying the flask, and also with the size and nature of the flask itself. With the same flask and with standard conditions of cleaning and drying, the blanks have been reasonably constant for a series of experiments. It has been our practice to do at least one blank determination to every two alcohol determinations.

A number of determinations on some of the common higher fatty alcohols were carried out satisfactorily using amyl ether and anisole Grignard reagents. Attempts were then made to estimate the hydroxyl groups in substances insoluble in these ethers or having only a very slight solubility. Determinations carried out on glucose and the dihydroxy-alcohol from the urine of pregnancy [Marrian, 1929] yielded the rather surprising result that no gas at all was evolved.

It was obviously necessary to dissolve such substances in some solvent that does not itself react with the reagent. Pyridine has been used by several workers in the past for this purpose, but there is some difference of opinion as to its suitability. Tanberg [1914], who was unable to obtain consistent results, criticises the use of pyridine. Zerewitinoff [1907] on the other hand claims that pyridine is well suited to the macro-method, giving consistent and accurate results if a few necessary precautions are observed in drying the pyridine and preparing the Grignard reagent. He suggests that the gas evolved when these precautions are not observed is ethane, produced according to the following equations:

1.

$$C_6H_5N + CH_3I \longrightarrow C_6H_5N\left\langle\begin{matrix}CH_3\\ I\end{matrix}\right.$$

2.


$$C_6H_5N\left\langle\begin{matrix}CH_3\\ I\end{matrix}\right. + \left.\begin{matrix}CH_3\\ I\end{matrix}\right\rangle Mg \longrightarrow C_6H_5N + MgI_2 + C_2H_6$$

and, therefore, if the Grignard reagent is quite free from methyl iodide, no gas is evolved.

With regard to the reaction between pyridine and Grignard's reagent we are in agreement with Zerewitinoff. When the reagent was prepared in the ordinary way, a large volume of gas was evolved when it was mixed with dry pyridine. If, however, the excess methyl iodide was first removed from the reagent by heating under reduced pressure, only a very small amount of gas was evolved with pyridine. The white solid compound that the reagent formed, even with dry pyridine, reacted completely but slowly with alcohols.

In spite of this the method proved to be a failure for micro-estimations, as the variations in the blanks caused by the introduction of another possible "wet factor" were so enormous. We thought at first that the method might be of use in distinguishing between the number of hydroxyl groups present in an alcohol, but even this is not too reliable, having an error up to 24 %.

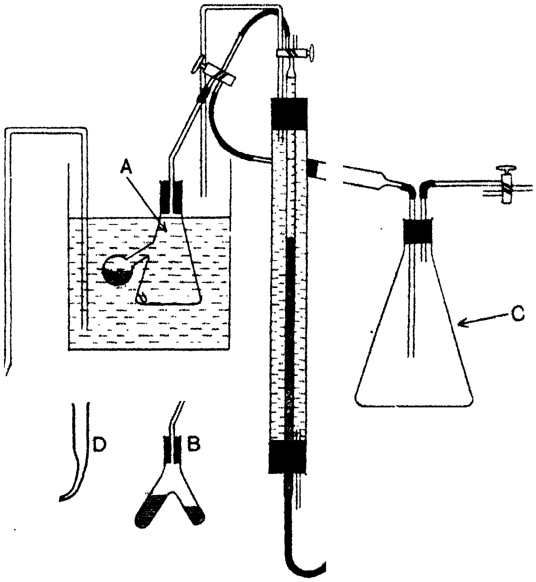


Fig. 1.

Experimental.

Apparatus.

The apparatus used was essentially the same as that previously described by Zerewitinoff (Fig. 1). The burette had a capacity of 5 cc., was 50 cm. long and graduated to hundredths of a cc. The flasks used were of two types (Fig 1 *A* and *B*). In all those experiments where pyridine was not employed, a flask of type *A* was used. Since, in the pyridine experiments, it was desirable to reduce the volume of the flask as much as possible to keep the blank to reasonable dimensions, a flask of type *B* was used having a capacity of about one-half of that of type *A*. This second type of flask has been found to be very satisfactory and is probably preferable for all determinations.

The apparatus was kept as far as possible at a constant temperature by running a rapid stream of tap water through the jacket surrounding the burette, and through the water-bath in which the reaction flask was placed. In order to have as much as possible of the apparatus at a constant temperature, the rubber connections between the flask and the burette were made as short as possible.

A three-way tap connected the circuit with a large flask (*C*) which was kept filled with dry nitrogen, so that if for any unforeseen reason, *e.g.* change in barometric pressure, the mercury level in the burette was too high when the initial reading was to be taken, more nitrogen could be run into the burette.

Preparation of Grignard's reagent.

(*a*) *Amyl ether reagent.* The amyl ether was dried by standing over sodium for at least a week. It was then redistilled. 4·5 g. pure magnesium ribbon were then heated in a dry flask on a sand-bath with 25 g. methyl iodide, 140 cc. amyl ether and a small crystal of iodine under a reflux condenser for about 15 minutes to start the reaction. The flask was then removed from the sand-bath and the reaction allowed to proceed by itself for about one hour. When the reaction slowed down, the flask was again heated for about an hour to complete the reaction. In the case of the reagent used for the pyridine experiments, the flask was heated under reduced pressure for about 15 minutes to remove the last traces of methyl iodide. The reagent prepared in this way remained clear and active for 2–3 months if kept in a tightly stoppered flask filled with dry nitrogen.

(*b*) *Anisole reagent.* The anisole reagent was prepared by exactly the same method as described above, substituting anisole for amyl ether. This reagent was far less stable than the amyl ether reagent. In the course of two or three weeks it deposited a heavy white precipitate and lost its power of reacting with alcohols.

Drying and cleaning of apparatus.

The exact technique of cleaning and drying the reaction flask and the pipettes used for measuring the reagents has been found to affect the magnitude of the blank. As a minimum blank is essential the methods of cleaning and drying have been varied to some extent in the different series of estimations carried out.

In series *A* (Table I), the flask, which was made of ordinary glass, was washed out in succession with hot water, dilute hydrochloric acid, water and finally acetone. It was then dried for at least 3 hours in an oven at 110°. Before use the flask was cooled in a desiccator over calcium chloride for 30 minutes.

In series *B* the flask, which was made of silica, was cleaned first with hot water and then boiled in a solution of chromic acid for at least an hour. The flask was then washed thoroughly with distilled water, heated to 110° for 30 minutes and finally to 170° for at least 3 hours over a dish of calcium chloride. As in series *A*, the flask was cooled for 30 minutes in a calcium chloride desiccator before use. The latter technique was adopted for series *C* (Table III) and series *D* (Table IV).

In each case the pipettes were cleaned and dried in the same way as the flask.

In preliminary experiments, we found that high results were invariably obtained unless special precautions were taken to ensure complete dryness of the substance under investigation. The bottle of substance was therefore left in a calcium chloride desiccator for several days before weighing the sample for the estimation. The sample, which was weighed into a small tube made by cutting down a hard-glass combustion tube to a length of about ½″, was then placed in a desiccator over P_2O_5 for at least 24 hours before use.

Drying the pyridine. The pyridine was allowed to stand over anhydrous barium oxide for a week and then distilled into a bottle containing fresh barium oxide.

Method of determination.

The exact method of carrying out the determination with the solid alcohol (without pyridine) was as follows. For 30 min. nitrogen was passed first through a calcium chloride tube and then through the apparatus, entering at the nitrogen reservoir and leaving at the rubber connection to which the flask was to be attached. The mercury in the burette was then adjusted to a suitable level by raising or lowering the reservoir slowly. The flask was then taken from the desiccator and rapidly blown out with a stream of dry nitrogen. The tube containing the substance was dropped into the flask and 3 cc. of Grignard reagent run into the side tube through a pipette and the special funnel shown in Fig 1 (*D*). The flask was then fitted to the greased stopper previously attached to the rubber connection of the burette. This was invariably carried out in the late afternoon, and the apparatus left overnight before taking the initial readings, so that any residual oxygen could be completely absorbed. The following morning, readings of the level of the mercury were observed at intervals of 10 min. after the temperature of the bath had become constant. When at least four consecutive readings were the same, the flask was taken from the bath, the reagent tipped out of the side-tube on to the alcohol, and the flask shaken vigorously for some minutes. Alternate shakings and readings were made until the mercury level was constant. The flask was then replaced in the water-bath to attain the correct temperature and readings were taken every few minutes until constant, the whole operation usually taking about 15 min.

The procedure in the experiments where the alcohol was dissolved in pyridine was different in several details. After removing the flask from the desiccator and blowing it out with nitrogen, one of us (*A*) placed the tube containing the alcohol in one leg of the flask while the other (*B*) measured out

3 cc. of Grignard reagent. While *B* was running the reagent into the other leg of the flask, *A* measured out 1 cc. of pyridine and then ran it into the leg containing the solid alcohol. *B* then connected the flask to the burette.

The following morning we invariably found that a crust of white solid about 2 mm. thick had formed on the surface of the reagent. We suggest that this was due to the withdrawal of final traces of water from the pyridine.

On tipping the Grignard's reagent into the leg of the flask containing the pyridine solution of the alcohol, a precipitate, presumably an addition compound of pyridine and the reagent, at once formed. The thickness of this precipitate caused the evolution of the gas to be extremely slow, and shaking at intervals for at least 40 min. was found to be necessary for the completion of the reaction. By tapping the leg of the flask and then shaking, the curd could be broken and poured from one leg of the flask to the other. This hastened the evolution of gas considerably. The reaction was considered to be complete when three consecutive readings at intervals of 5 min. were the same.

CALCULATIONS AND RESULTS

The volume of methane evolved in each experiment was corrected to 0° and 760 mm. From this the average corrected blank for the particular series was subtracted. The percentage hydroxyl in the compound could then be calculated from the formula:

$$\% \text{ OH} = \frac{17 \times 100 \times V}{22380 \times W},$$

where 17 is the molecular weight in g. of the hydroxyl group, *V* the corrected volume in cc., and *W* the weight in g. of substance taken. The results are shown in Tables I, II, III and IV.

Table I. *Amyl ether reagent.*

Blanks (at N.T.P.) 0·14 cc., 0·11 cc., 0·13 cc., 0·11 cc., 0·10 cc. Mean 0·12 cc.

Substance	Weight g.	cc. CH_4 at N.T.P.	cc. CH_4 corrected for blank	% OH Found	% OH Theoretical	% Error
Octadecyl alcohol	0·0113	1·04	0·92	6·18	6·29	−1·7
Octadecyl alcohol	0·0102	0·94	0·82	6·10	6·29	−3·0
Octadecyl alcohol	0·0102	0·96	0·84	6·25	6·29	−0·7
Octadecyl alcohol	0·0100	0·98	0·86	6·53	6·29	+3·8
Batyl alcohol	0·0123	1·75	1·63	10·06	10·88	−7·5
Batyl alcohol	0·0122	1·78	1·67	10·39	10·88	−4·5
Cholesterol	0·0098	0·67	0·55	4·27	4·40	−2·9

Table II. *Amyl ether reagent.*

Blanks (at N.T.P.) 0·09 cc., 0·08 cc., 0·05 cc., 0·07 cc., 0·09 cc. Mean 0·08 cc.

Substance	Weight g.	cc. CH_4 at N.T.P.	cc. CH_4 corrected for blank	% OH Found	% OH Theoretical	% Error
Octadecyl alcohol	0·0110	0·97	0·89	6·14	6·29	−2·4
Octadecyl alcohol	0·0101	0·91	0·83	6·24	6·29	−0·8
Hexadecyl alcohol	0·0108	1·06	0·98	6·89	7·08	−2·7
Hexadecyl alcohol	0·0102	1·03	0·95	7·08	7·08	0·0
Hexadecyl alcohol	0·0116	1·20	1·12	7·34	7·08	+3·6
Cholesterol	0·0110	0·68	0·60	4·15	4·40	−5·7
Cholesterol	0·0107	0·73	0·66	4·69	4·40	+6·6

Table III. *Anisole reagent.*

Blanks (at N.T.P.) 0·09 cc., 0·07 cc., 0·04 cc., 0·04 cc., 0·07 cc., 0·04 cc. Mean 0·06 cc.

Substance	Weight g.	cc. CH_4 at N.T.P.	cc. CH_4 corrected for blank	% OH Found	% OH Theoretical	% Error
Cholesterol	0·0105	0·64	0·58	4·19	4·40	−4·8
Cholesterol	0·0109	0·67	0·61	4·25	4·40	−3·4
Dilauryl alcohol	0·0106	0·72	0·66	4·73	5·00	−5·4
Dilauryl alcohol	0·0099	0·77	0·71	5·44	5·00	+8·8
Octadecyl alcohol	0·0092	0·78	0·72	5·94	6·29	−5·5

Table IV. *Amyl ether reagent. Substance dissolved in 1 cc. pyridine.*

Blanks (at N.T.P.) 0·40 cc., 0·17 cc., 0·38 cc., 0·25 cc., 0.27 cc., 0·09 cc., 0·22 cc., 0·26 cc. Mean 0·26 cc.

Substance	Weight g.	cc. CH_4 at N.T.P.	cc. CH_4 corrected for blank	% OH Found	% OH Theoretical	% Error
Cholesterol	0·0111	1·00	0·74	5·06	4·40	+15·0
Cholesterol	0·0082	0·81	0·55	5·09	4·40	+15·7
Cholesterol	0·0076	0·75	0·49	4·89	4·40	+11·1
Cholesterol	0·0096	0·69	0·42	3·32	4·40	−24·5
Hexadecyl alcohol	0·0082	0·89	0·63	5·83	7·03	−17·1
Hexadecyl alcohol	0·0110	1·16	0·90	6·21	7·03	−11·7
Hexadecyl alcohol	0·0092	0·99	0·73	6·02	7·03	−14·4
Quinol	0·0128	4·39	4·13	24·49	30·91	−20·7
Quinol	0·0026	1·16	0·90	26·27	30·91	−15·0

SUMMARY.

1. A micro-method for estimating the percentage of hydroxyl in the higher fatty alcohols, based on the macro-method of Hibbert and Sudborough [1904] is described.

2. Results accurate to ± 8 % are obtained when the alcohol is readily soluble in the ether of the Grignard reagent used.

3. The use of pyridine to dissolve alcohols which are not soluble in the ether of the reagent is not recommended, the results being very unreliable owing to a variable blank.

The authors wish to express their thanks to Prof. J. C. Drummond for his interest in this work. The expenses incurred were defrayed by a grant to one of us (G. F. M.) from the Medical Research Council, to whom we wish to express our gratitude.

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LXXXV. NATURAL ANTHOCYANIN PIGMENTS.

I. THE MAGENTA FLOWER PIGMENT OF *ANTIRRHINUM MAJUS.*

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(*Received May 1st, 1930.*)

FROM her study of the Mendelian factors for flower colour in *Antirrhinum majus*, Wheldale [1907, 1914] showed that, on crossing white with yellow or ivory varieties, plants bearing red or magenta flowers are produced. This led her to suggest that the red and magenta anthocyanin pigments might be derived from the yellow and ivory flavones, and that the white varieties, from which these pigments are absent, might carry the factors which act on the flavones to form anthocyanin.

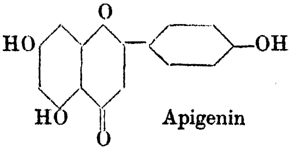
With a view to testing this hypothesis, Wheldale [1913] commenced an investigation of the chemistry of these four pigments. With Bassett [1913, 1914, 1] she isolated the yellow and ivory flavone pigments and identified them as glucosides of luteolin and apigenin. The yellow flowers contained glucosides of both these pigments, while apigenin alone was present in the ivory variety. From the red and magenta flowers they isolated two amorphous pigments of high molecular weight and uncertain constitution, which were apparently uncombined with any acid [Wheldale and Bassett, 1914, 2].

At this time Willstätter and Everest [1913] published an account of the isolation of the blue anthocyanin from *Centaurea cyanus* (cornflower). In this classical paper, they revealed the constitution and true nature of the anthocyanins, showing that they could be isolated by means of their stable crystalline oxonium salts. Unfortunately, this communication, which revolutionised the methods employed in the extraction and isolation of these pigments, was published too late to be of any use to Wheldale and Bassett, and the question of the relationship between these co-existing flavones and anthocyanins remained unanswered.

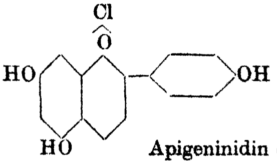
More recent research has still left the question open [Onslow, 1925] as to whether anthocyanins are derived directly from flavones or not: it is possible for instance that they are formed independently from the same parent substances.

With a view to throwing more light upon the interrelationships between flavones and anthocyanins in the same varieties, a further attempt has been made to isolate and identify the red and magenta pigments of *Antirrhinum majus*. The pure crystalline chloride of the magenta pigment *antirrhinin* has been isolated by a process based on the methods of Willstätter and his co-workers. The preparation of the red pigment is to be attempted next.

If there were any evidence for the direct origin of anthocyanins from flavones in the plant, the genetical relationships would point to apigenin as the parent substance of this magenta pigment. On crossing an ivory variety with a white of certain origin, a magenta offspring is produced. In this case the flavone apigenin is the only pigment present in either parent, and only this flavone and magenta anthocyanin are to be found in the offspring. If Wheldale's original hypothesis had been correct, some simple relationship should be apparent between the structures of these two pigments. Wheldale had originally suggested that the reaction occurring on the formation of anthocyanins would be one of oxidation. After the elucidation of the constitution of the anthocyanins, and the production by reduction, *in vitro,* of the anthocyanidin cyanidin from the flavone quercetin [Willstätter and Mallison, 1914], Everest [1918] supported the view that the process *in vivo* was one of reduction. If this second theory is correct, the magenta anthocyanin should prove to be apigeninidin, a pigment which has not yet been found to occur naturally.

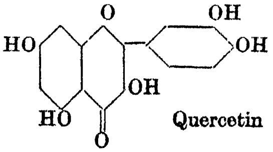


Apigenin

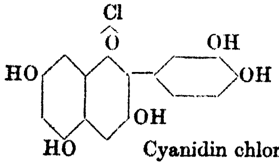


Apigeninidin chloride

The analysis of antirrhinin chloride, however, has shown it to be a 3-rhamnoglucoside of cyanidin chloride, which is closely related, structurally, to the flavonol quercetin and not to apigenin.



Quercetin



Cyanidin chloride

Since cyanidin contains one more molecule of oxygen than apigenin, the connection between these two pigments cannot be one of reduction, nor can one explain their relationship by any simple oxidative process. Wheldale and Bassett did not identify the sugar residues attached to the flavones of *Antirrhinum,* so that it is not yet known whether there is any correlation between the carbohydrate portions of these co-existing pigments.

The determination of the identity of antirrhinin therefore throws no further light upon the nature of a relationship, either of oxidation or reduction, between flavones and anthocyanins, which has been indicated by the study of the Mendelian factors.

In only four other cases have the constitutions of the co-existing flavones and anthocyanins been determined.

The red rose contains cyanin [Willstätter and Nolan, 1914], together with the corresponding flavone quercetin [Karrer and Schwarz, 1928], while the author (unpublished work) has recently identified a similar anthocyanin in brown *Cheiranthus cheiri* (wallflower), from which quercetin and its monomethyl ether, *iso*rhamnetin, have already been isolated [Perkin and Hummel, 1896]. Thus in these two flowers the constitution of the pigments favours the theory of their interconversion *in vivo.*

The significance of the results in the case of *Viola tricolor* is at present not very clear, since Everest [1919] claims that myricetin is present in this flower together with the quercetin and delphinidin identified by Mandelin, and later by Perkin [1902] and by Willstätter and Weil [1916]. His claim, however, is not based on analytical results.

The flavone kaempferol [Perkin and Wilkinson, 1902] and the anthocyanin delphinin [Willstätter and Mieg, 1914] have been isolated from *Delphinium consolida*: thus here, as in *Antirrhinum*, no close relationship is apparent.

The information derived from the study of the constitution of these flavones and anthocyanins is therefore conflicting. There is no conclusive evidence that the plant produces the latter from the former, or that they are both formed by separate but parallel syntheses from the same parent substance.

The flowers used for this investigation were those of the deep crimson varieties, *Purple King* (Carter), *Purple Queen* (Ryder), and *Giant Crimson* and *Dwarf Crimson* (Sutton). They all presumably contained the magenta anthocyanin together with luteolin and apigenin. The plants, which were grown on a plot of land kindly lent to me by Miss E. R. Sanders of Newnham College, were planted and tended by the staff of the Cambridge Botanic Gardens.

The isolation of the pigment was carried out by a modification of Willstätter and Zollinger's [1916] method for the preparation of keracyanin chloride from the cherry. Dilute methyl alcoholic hydrochloric acid was used for the first extraction, since glacial acetic acid extracts contained large amounts of flavone which formed a gel, and made filtration very difficult. Owing to the flavone present even in the alcoholic extracts, great difficulty was experienced at first in obtaining the chloride of the pigment in a pure enough state for crystallisation, but this was at length achieved.

Purification of the pigment by means of the crystalline picrate was not possible in this case, owing to the extreme solubility of this salt. Use was therefore made of the insoluble lead salt. This was precipitated from the crude alcoholic extracts, and then dissolved in glacial acetic acid, in which the lead salts of flavones and other contaminating substances are not soluble.

The yield of pure pigment was small in proportion to the large amount of material used, but, as flowers were not lacking, it was found to be more economical in labour and reagents to start with large quantities, and subsequently to discard mother-liquors which still retained appreciable amounts of pigment.

The pure chloride of antirrhinin was finally isolated in four hydrated forms. These differed in colour and water of crystallisation, and each of them possessed a characteristic crystalline form.

The chloride of the sugar-free pigment was identified as cyanidin chloride by means of its appearance, general properties and reactions, combustion analyses and products of alkali fission. In all these respects, perfect agreement was found with the account of this pigment given by Willstätter and Everest [1913].

It has also been possible to compare the pigment from *Antirrhinum* with a sample of pure synthetic cyanidin chloride which was very kindly sent to me by Prof. R. Robinson. They showed a close resemblance both in their absorption spectra and behaviour in a range of buffered solutions. This latter method, which has been introduced by Robertson and Robinson [1929], is an excellent and reliable means of comparing and characterising anthocyanins and anthocyanidins.

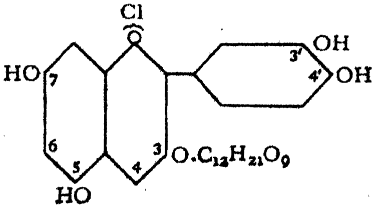
The identification of the sugar residue as a combination of a molecule each of rhamnose and glucose was determined by means of the distribution number, and qualitative and quantitative analyses of the sugars produced on hydrolysis.

The position of the sugar residue at position 3 in the antirrhinin molecule is indicated by the violet colour reaction given with sodium carbonate. If the substitution were at 5 or 7, the colour reaction would be either pure blue or violet blue, according to whether the hydroxyls at 7, 3, 3′ and 4′, or at 5, 3, 3′ and 4′, were unsubstituted. This decision is strengthened by the fact that, after hydrolysis of the glucoside, one obtains a pure blue reaction with the resulting cyanidin chloride from which the rhamno-glucosidal residue at position 3 has been removed. The validity of these colour reactions as a test for the position of the sugar residue has been studied by Robinson and his co-workers during their extensive syntheses, first of pyrylium compounds [Pratt and Robinson, 1925], and later of substituted anthocyanidins, and even of anthocyanins themselves [Robertson and Robinson, 1927, 1928].

Keracyanin, the pigment of the fruit of the cherry, not only has a similar constitution to that of antirrhinin, but appears from its reactions to be identical. Keracyanin chloride was isolated by Willstätter and Zollinger [1916] who describe two hydrates which resemble the red and brown hydrates of antirrhinin chloride.

The pigment isolated by Karrer and Widmer [1927] from the fruit of *Sambucus niger* (elderberry), and named by them sambucin, is also a rhamnoglucoside of cyanidin, probably together with traces of some other pigment. Many of the reactions of sambucin chloride resemble those of antirrhinin chloride, but no mention is made of the existence of different hydrated forms, and the crystalline picrate, which they isolated without any difficulty, must have been much less soluble than that of antirrhinin.

Antirrhinin chloride may be represented by the following formula:



EXPERIMENTAL.

The extraction and purification of the pigment.

The pigment was extracted with 2 % methyl alcoholic hydrochloric acid (8 litres) from about 10 kg. of fresh crimson flowers, from each of which both calyx and pistil had been removed. After standing for 2 days, the first extract was separated off, and the residue squeezed and re-extracted with a further 7 litres of acid-alcohol. Even after these two extractions some pigment was still retained.

A saturated aqueous solution of lead acetate was slowly added to the filtered extract until there was an excess. Lead chloride precipitated first; the solution then turned from red to purple, green, and then dark blue; finally the pigment was precipitated, together with the flavones, as a dark blue lead salt.

The mixture was allowed to stand for 2 days, by which time most of the pigment had been precipitated. After decantation of the red-brown mother-liquor, the precipitate was filtered, washed with a small amount of methyl alcohol, and sucked as dry as possible. A certain amount of pigment remained in the mother-liquor. Addition of ethyl alcohol precipitated some of this, and more came down on standing, but it was not possible to recover it all.

The moist, dark blue precipitate from two litres of alcoholic extract was extracted with successive amounts of glacial acetic acid, about 300 cc. being used in all. The extraction was incomplete, even on frequent rubbing and stirring. This was probably due to the retention of the pigment by the lead chloride. After standing for some hours, the dark-red acetic acid extract was again filtered from further impurities which had settled out.

On the addition of two volumes of purified ether, the lead salt was precipitated in dark blue granules, which were filtered off, washed with ether, dried and ground. The filtrate, which was green in colour, retained much of the impurity.

The rest of the original lead precipitate was purified in the same manner, about 100 g. of nearly pure lead salt being obtained in all. In some cases the ether precipitated the salt as a gummy mass, which absorbed water from the air. It was partially dried by rubbing with absolute alcohol and filtering. This deliquescence was probably due to the impurities present. It was noted, later on, that precipitation of the impure chloride of the pigment with ether generally gave a dry powdery residue, which quickly became sticky, but as purification proceeded, the pigment obtained was much less deliquescent.

11·0 g. of the finely ground lead salt were dissolved in a minimum of 5–10 % hydrochloric acid in methyl alcohol, and the lead chloride was filtered off. (If left to stand, a gel was formed due to flavone impurity.) One-third the volume of propyl alcohol was next added and the solution left overnight; the impurities which came down were filtered off, and, on addition of purified ether, the pigment was recovered as a flocculent, scarlet precipitate. The purity of the bright red, gummy mass, obtained on filtering, was tested by hydrolysing a small amount. The sugar-free pigment which separated was mostly amorphous, but a few crystals were found on examination under the microscope.

The glucoside was next dissolved in a few cc. of warm methyl alcohol, made 20 % acid with hydrochloric acid and left to stand overnight. The chloride of the pigment was precipitated as a dark brown amorphous, or microscopically crystalline, substance, and a sample now gave, on hydrolysis, homogeneous needle crystals of the non-glucosidal pigment.

The glucoside was filtered, washed quickly with 20 % methyl alcoholic acid and dried by suction. The yield was 1·9 g.

The mother-liquor still retained much of the pigment. Part of this was later isolated, but great difficulty was experienced, as the impurities still present kept the pigment from precipitating.

The glucoside was recrystallised twice by solution in warm methyl alcohol, and addition of one-fifth the volume of concentrated acid. On standing overnight, the precipitation of the pigment as golden brown crystals with a bronze reflex was almost complete.

1·4 g. of pure antirrhinin chloride were thus obtained, and this method of purification was repeated on the rest of the crude pigment.

The glucosidal pigment, antirrhinin chloride $C_{27}H_{31}O_{15}Cl$.

Antirrhinin chloride can be prepared in at least four different hydrated forms. The appearance, composition, method of preparation and characteristics are given in the accompanying table:

Hydrate	Appearance	Properties	Method of preparation
Yellow hydrate $C_{27}H_{31}O_{15}Cl, 1\frac{1}{2}H_2O$	Orange-yellow ppt. of fine, pale yellow, ill-defined needles. (See Fig. 1). Yellow powder when dry, turning brick-red after several weeks	V. insol. in cold water In hot water dissolves and isomerises instantaneously Barely soluble in cold or hot HCl solution (except in 3 %) Fairly soluble in cold alcohol, more soluble hot Insol. in neutral amyl alcohol, but more soluble when acidified	Precipitated from warm methyl alcohol solutions on standing. Precipitation not complete
Fan hydrate $C_{27}H_{31}O_{15}Cl, 2\frac{1}{2}H_2O$	Dark purple-brown ppt. of feathery clusters of crystals resembling Japanese fans. (See Fig. 2)	Soluble in cold and hot water, alcohol and aqueous hydrochloric acid	Precipitated from strong (20 %) aqueous HCl; never from acid-alcohol. Often contaminated by the brown hydrate
Red hydrate $C_{27}H_{31}O_{15}Cl, 3\frac{1}{2}H_2O$	Scarlet flocculent ppt. of fine needles. Brick coloured powder when dry. (See Fig. 3)	S. soluble in cold water. More soluble on heating Soluble in alcohol and in aqueous hydrochloric acid	Precipitated from aqueous 5 % HCl. A mixture of the red and brown hydrates separates from 10 % acid
Brown hydrate $C_{27}H_{31}O_{15}Cl, 4?H_2O$	Brown crystalline ppt. with bronze reflex, composed of pale grey-brown leaflets in clusters. (See Fig. 4) Marks paper with a purple streak	V. soluble in cold water and alcohol V. soluble in dil. HCl, but decreasingly sol. in higher concs. More soluble on heating	Precipitated from 10 % acid methyl alcoholic solutions on cooling and standing

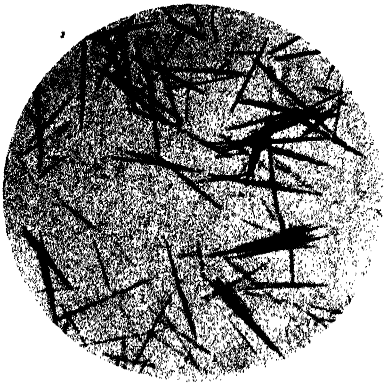


Fig. 1. The yellow hydrate.

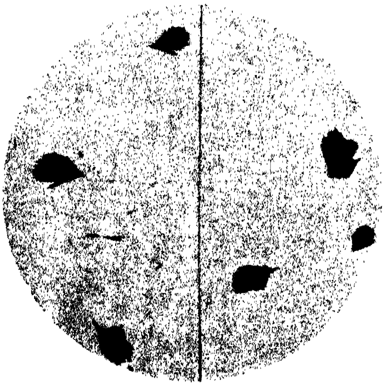


Fig. 2. The fan hydrate.

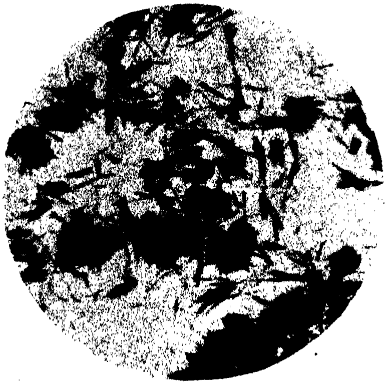


Fig. 3. The red hydrate.

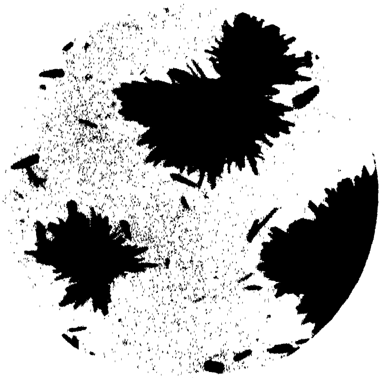


Fig. 4. The brown hydrate.

The following are the chief reactions of antirrhinin chloride:

Ferric chloride.	Fairly stable, good blue in alcohol. Violet, and less stable, in water.
Sodium acetate.	Violet colour.
Potassium acetate.	Plum-coloured solution.
Sodium carbonate.	Blue-violet to red-violet.
Sodium hydroxide.	Sapphire blue to green-blue.
Lead acetate.	Blue precipitate.
Perchloric acid.	Dissolves on warming. On standing, pale red-brown crystals of perchlorate separate in sheaves.
Picric acid.	Very soluble, no crystalline picrate separates.
7 % *sulphuric acid.*	Soluble on heating. On standing, clusters of fine orange needles separate.
Fehling's solution.	Reduced.

The violet reaction with sodium carbonate indicates that the sugar-residue is attached to the molecule at position 3.

A solution of the colourless *pseudo*-base is easily produced on adding the pigment to a large volume of hot water, and heating. If acid is added to this solution, it reddens again, even in the cold. The purple colour base is partially precipitated when a concentrated water solution of the glucoside is heated.

A Zeisel estimation showed the complete absence of methoxyl groups.

The colour reactions and stability of antirrhinin chloride in a series of buffered solutions were determined by Robertson and Robinson's method.

The 17 buffer solutions, starting from p_H 3·2 and each increasing by 0·6 p_H, were prepared by the addition of suitable amounts of 0·2 *N* sodium hydroxide (CO_2-free) to 50 cc. of "Universal Buffer Solution" supplied by the British Drug Houses. The p_H values up to 11·0 were calculated by means of the formula supplied. Equal amounts (10 cc.) of each buffer were placed in a series of boiling-tubes, 1 cc. of an alcoholic solution of the pigment (25·0 mg. in 100 cc. 98 % alcohol) was added to each tube from a burette, and the tubes well shaken and examined, both at the moment of mixing, and at intervals during the next 48 hours. The results are given in the following table:

	p_H	Colour reaction and stability
10 % HCl		Salmon red
1 % HCl		Pink
I	3·2	Pink, fades quickly, but still pale pink after 3 days
II	3·8	Pink, fades less quickly
III	4·4	Pink, fades slowly
IV and V	5·0 and 5·6	Mauve pink, fades more slowly
VI	6·2	Mauve pink, more stable
VII	6·8	Brownish pink, fades slightly
VIII and IX	7·4 and 8·0	Brownish cherry, fades slightly
X to XII	8·6 to 9·8	Brownish cherry, very stable even after 16 hours
XIII and XIV	10·0 and 11·0	Bordeaux red, very stable even after 16 hours
XV		Cherry with blue tinge
XVI		Permanganate colour, slowly destroyed, pale brownish pink in 16½ hours
XVII		Deep violet, fades slowly
10 % NaOH solution		Pure blue, very unstable

The following figures were obtained for the loss in weight of the four hydrates, on removal of their water of crystallisation, by drying *in vacuo* over phosphorus pentoxide at 100° and 105°.

The 'yellow' hydrate	
I. 77·8 mg. 'yellow' hydrate lost 1·8 mg. on drying at 100°	1·93 %
72·5 mg. 'yellow' hydrate (dried at 100°) lost 1·7 mg. on drying at 105°	2·35 %
Total loss in weight on drying at 105°	4·28 %
II. 179·1 mg. yellow hydrate lost 8·1 mg. on drying at 105°	4·52 %
Calculated for $C_{27}H_{31}O_{15}Cl, 1\frac{1}{2}H_2O$	4·12 %
The 'fan' hydrate	
271·4 mg. 'fan' hydrate lost 17·7 mg. on drying at 105°	6·52 %
4·837 mg. 'fan' hydrate lost 0·320 mg. on drying at 105°	6·5 %
Calculated for $C_{27}H_{31}O_{15}Cl, 2\frac{1}{2}H_2O$	6·52 %
The 'red' hydrate	
53·4 mg. 'red' hydrate lost 4·8 mg. on drying at 105°	9·0 %
34·0 mg. 'red' hydrate lost 3·0 mg. on drying at 105°	8·8 %
Calculated for $C_{27}H_{31}O_{15}Cl, 3\frac{1}{2}H_2O$	9·0 %
The 'brown' hydrate	
0·0790 g. 'brown' hydrate lost 0·0074 g. on drying	9·36 %
Calculated for $C_{27}H_{31}O_{15}Cl, 4H_2O$	10·2 %

Micro-analyses of the substances after drying for carbon, hydrogen and chlorine gave the following figures:

4·821 mg. 'yellow' hydrate gave 8·960 mg. CO_2 and 2·18 mg. H_2O
5·20 mg. 'yellow' hydrate gave 1·09 mg. AgCl
4·517 mg. 'fan' hydrate gave 8·250 mg. CO_2 and 2·03 mg. H_2O
6·963 mg. 'fan' hydrate gave 1·623 mg.

The percentage compositions are therefore:

	C	H	Cl
'Yellow' hydrate	50·6	5·04	5 13
'Fan' hydrate	49·8	4·99	5·76
Calculated for $C_{27}H_{31}O_{15}Cl$ (cyanidin rhamnoglucoside)	51·39	4·92	5·62

The combustion analyses for the C and H content of antirrhinin chloride and the sugar-free pigment were carried out by Dr Ing. A. Schoeller of Berlin-Schmargendorf, and those for chlorine by A. Colwell of this laboratory.

The sugar-free pigment, cyanidin chloride, $C_{15}H_{11}O_6Cl$, H_2O.

The sugar-free pigment was obtained by hydrolysis of antirrhinin chloride with boiling 20 % hydrochloric acid for 4 minutes. After 2 minutes pale red-brown crystals began to separate in long needles (see Fig. 5). On cooling, filtering, washing with a little 20 % acid, and drying, a reddish chocolate-brown powder was obtained.

If dissolved in methyl alcohol, an equal volume of 10 % aqueous hydrochloric acid added, and left to evaporate, the pigment precipitates almost quantitatively in well-formed crystals. These are not long and tapering as before, but are more sturdy and collect in stars and clusters.

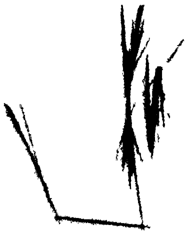


Fig. 5. Cyanidin chloride from Antirrhinum.

When the pigment is dissolved in methyl alcohol, acid added and the alcohol boiled off, the salt reprecipitates in a curious symmetrical S-shaped form with curled offshoots.

The sugar-free pigment is slightly soluble in cold water, giving a mauve-red solution, which on heating turns purple, and then isomerises. It is difficultly soluble in aqueous hydrochloric acid of all strengths: on heating, it has a maximum solubility in 0·3 % acid, and in the cold in 0·03 % acid, a colour base being precipitated if this solution is heated. A hot saturated solution in 5·0 % acid does not contain much pigment, but on cooling even this is precipitated in fine needles.

The pigment is very soluble in methyl and ethyl alcohol, giving beautiful red-violet solutions which turn an intense cerise colour on acidification.

If water and not acid be added, a precipitate of the purple colour-base is immediately formed, and a green sheen appears on the sides of the tube. This behaviour is typical of cyanidin chloride.

Solutions in amyl alcohol are violet, and isomerise on standing. On acidification with an equal quantity of dilute acid, the alcohol layer turns crimson. On addition of alkali, the pigment passes into the water layer and turns blue. An acetone solution is violet.

Ethyl acetate dissolves the pigment to a small extent, giving a crimson solution on acidification, and a clear green-blue on addition of sodium hydroxide.

The chief reactions are:

Ferric chloride.	Blue colour in alcohol. Purple on addition of water. Unstable on addition of excess of reagent.
Sodium carbonate.	Violet and then pure blue.
Sodium hydroxide.	Clear green-blue, unstable.
Sodium acetate.	Red-purple solution in alcohol, purple in water.
Potassium acetate.	Dichroic red-blue in alcoholic solution. Colour base precipitated on standing.
Lead acetate.	Blue precipitate of the lead salt.
Perchloric acid.	Very soluble. No crystals of perchlorate are formed.
Picric acid.	Soluble on warming. Burr-like clusters of orange-red picrate crystals separate on standing.
7 % *sulphuric acid.*	Slightly soluble on heating. Brown hair-fine needles of sulphate separate on standing.
Fehling's solution.	Reduced slowly on heating.

It did not melt when heated up to 300°.

Acid amyl alcohol solutions gradually lose their colour on standing in the light. In a few days most of the pigment is destroyed.

A purple colour-base can be prepared by heating strong aqueous solutions of the pigment, from which it is quantitatively precipitated as an amorphous flocculent precipitate.

A solution of the colourless *pseudo*-base is obtained on adding an alcoholic solution of the pigment to a large volume of hot water. Addition of acid will not reproduce the red oxonium salt in the cold, but will do so on heating—a phenomenon peculiar to cyanidin chloride.

On fusion of the pigment with 50 % potassium hydroxide, small amounts of the breakdown products were isolated and found to be phloroglucinol and protocatechuic acid. The phloroglucinol was identified by means of its characteristic orange-red diazobenzene compound, the red colour given with a pine shaving and concentrated hydrochloric acid, the brick-red phloroglucide of methylfurfuraldehyde precipitated after heating with rhamnose and 20 % hydrochloric acid, and the darkening due to oxidation on addition of potassium hydroxide. The protocatechuic acid gave a green colour with ferric chloride followed by the very characteristic violet changing to violet-red on addition of weak sodium carbonate solution.

The crystalline chloride of the sugar-free pigment was dried at 105° *in vacuo* over phosphorus pentoxide, and eventually lost all its water of crystallisation. The last traces were very difficult to remove, and the whole amount was quickly absorbed again on standing in air. Combustion analyses were therefore carried out on the crystalline and the dried pigment. The materials used in the third and fourth analyses were dried and undried samples of the same specimen. In the fourth analysis the agreement with the calculated values was better than that of the first three: a fact which points to the retention of a small proportion of water of crystallisation by the dried pigment.

On analysis, the following values were obtained:

0·1462 g. chloride dried at 105° lost 0·0082 g. = 5·58 %
0·1784 g. chloride dried at 105° lost 0·0092 g. = 5·16 %
$C_{15}H_{11}O_6Cl, H_2O$ dried at 105° would lose 5·28 %

I (*a*). 4·854 mg. chloride dried at 105° gave 9·51 mg. CO_2 and 1·73 mg. H_2O
I (*b*). 5·904 mg. chloride dried at 105° gave 2·596 mg. AgCl
II. 4·009 mg. chloride dried at 105° gave 1·756 mg. AgCl
III. 4·682 mg. chloride dried at 105° gave 9·36 mg. CO_2 and 1·64 mg. H_2O
IV. 4·666 mg. chloride (air-dried) gave 8·995 mg. CO_2 and 1·70 mg. H_2O

The percentage compositions are therefore:

		C	H	Cl
By experiment.	Sample I	54·38	3·72	10·87
	Sample II	—	—	10·83
	Sample III	54·55	3·90	—
	Sample IV (air-dried)	52·57	4·05	—
Calculated for $C_{15}H_{11}O_6Cl$		55·81	3·42	11·00
Calculated for $C_{15}H_{11}O_6Cl, H_2O$		52·94	3·82	—

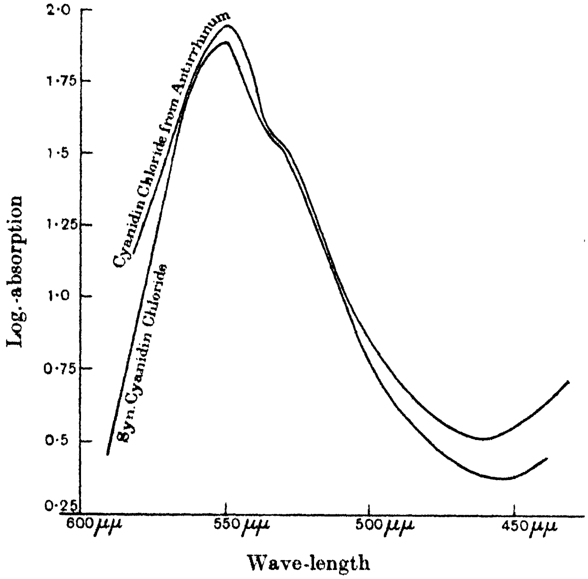


Fig. 6.

The sugar-free pigment was further identified as cyanidin chloride by means of its colour reactions, power of isomerisation and stability in the series of buffers. On comparing the pigment side by side with a sample of pure cyanidin chloride, the behaviour of these two pigments over the whole range was almost identical. Above p_H 8·6 the colour given by cyanidin chloride was slightly more violet, but this was only noticeable on very careful comparison. The reactions were similar to those described for cyanidin chloride by Robertson and Robinson.

The absorption spectrum of the chloride of the sugar-free *Antirrhinum* pigment was also compared with that of a synthetic sample of cyanidin chloride. From the readings taken with a spectrophotometer, the log. absorption curves were plotted over the visible spectrum (see Fig. 6), and were found to be very similar. The maximum absorption of the dark band in the green region is, in each case, at a wave-length of $550\mu\mu$. Another band in the violet is also indicated. Acid (HCl) alcoholic solutions were used of such a strength that the maximum value found for the log. absorption lay between 1·75 and 2·0. Since the concentration of the synthetic cyanidin chloride solution was less than that of the *Antirrhinum* pigment, the curves, though parallel, were not identical.

The sugar residue.

The identification of the glucosidal residue of antirrhinin was carried out by means of the "distribution number," and by qualitative tests and quantitative estimations of the free reducing sugars and cyanidin chloride liberated on hydrolysis.

The "distribution number" for antirrhinin chloride was found by dissolving 0·005 g. of pigment in 25 cc. 5 % hydrochloric acid and shaking with two successive amounts of 25 cc. pyridine-free amyl alcohol. The amount of pigment extracted in each case was measured colorimetrically, by comparison with a standard made from 0·005 g. of pigment, dissolved in 1 cc. methyl alcohol, and made up to 25 cc. with amyl alcohol, a trace of acid being added to prevent isomerisation.

The mean value for the two extractions was 8·07, which compared well with the values for other rhamnoglucosides, which all lie between 6·5 and 10.

Qualitative sugar tests were carried out on the hydrolysis mixture, after filtering off the crystalline sugar-free pigment and removing any remaining traces of colour by extraction with amyl alcohol. A distinct smell of methylfurfuraldehyde could be detected during the hydrolysis. This is typical of rhamnoglucosides.

A portion of the filtrate was tested for pentose by the orcinol test. The solution turned pink (as for rhamnose) on boiling with orcinol and hydrochloric acid, and, on adding a drop of ferric chloride gave a green which was extracted by amyl alcohol. No blue precipitate (as for ordinary pentose) was obtained on boiling. The green colour was similar to that found with rhamnose, and was not of the vivid blue-green obtained with arabinose.

On hydrolysing for some time with strong hydrochloric acid, the distillate gave no pink with aniline acetate paper (therefore not normal pentose), but, on adding phloroglucinol, gave the characteristic yellow colour, and, on standing, a brick-red precipitate of the phloroglucide of methylfurfuraldehyde.

After neutralising the original filtrate with lead carbonate, filtering, and removing excess lead with hydrogen sulphide, the mixed osazone was prepared and compared with that from a mixture of pure rhamnose and glucose.

Quantitative estimations of the percentage of cyanidin chloride and reducing sugars obtained on hydrolysis of antirrhinin chloride were next carried out.

Weighed quantities of antirrhinin chloride (dried at 105°) were dissolved in 3 to 5 cc. of 1 % hydrochloric acid, and hydrolysed by boiling with 10 to 20 cc. 20 % acid for 2½ to 3 minutes. After standing in the ice-chest until the mother-liquor was barely pigmented, the crystalline cyanidin chloride was collected on a weighed Pregl glass filter, washed with a small amount of 20 % acid, dried *in vacuo* at 105° over P_2O_5, and weighed. One yield was air-dried, an allowance being made, in the calculation of the percentage, for the presence of one molecule of water.

After extraction of the remaining pigment with amyl alcohol, and subsequent removal of traces of the latter with pure ether, the filtrate was nearly neutralised with a small quantity of sodium hydroxide, and made up to a definite volume. The sugars were then estimated both polarimetrically and by Bertrand's method, it being assumed that rhamnose and glucose were present in equimolecular proportions. The polarimetric values obtained confirmed the identification of the hexose as glucose. Accurate polarimetric readings were taken by Mr B. Woolf of this laboratory, to whom I am much indebted.

The rhamnose content was estimated by the phloroglucide method [Ellett and Tollens, 1905], an aqueous solution of a weighed quantity of the glucoside being treated directly with 12 % aqueous hydrochloric acid.

I. 31·1 mg. antirrhinin chloride gave 16·80 mg. cyanidin chloride
31·1 mg. „ „ 13·59 mg. rhamnose + glucose (26·52 mg. Cu)
II. 187·7 mg. „ „ 104·20 mg. cyanidin chloride (air dried)
187·7 mg. „ „ 82·30 mg. rhamnose + glucose (161·7 mg. Cu)
III. 490·2 mg. „ „ 262·50 mg. rhamnose + glucose (50 cc., $l = 4$ dm., $\alpha_D = +0·65°$)
IV. 135·8 mg. „ „ 65·02 mg. rhamnose + glucose (25 cc., $l = 2$ dm., $\alpha_{Hg} = +0·19°$)
V. 231·4 mg. „ „ 62·40 mg. rhamnose (33·00 mg. phloroglucide)

The percentage yields are therefore:

	% cyanidin chloride	% combined sugars	% rhamnose
By experiment I	54·00	43·75	—
II	53·00	43·80	—
III	—	53·30	—
IV	—	47·80	—
V	—	—	27·00
Calculated for $C_{27}H_{31}O_{15}Cl$	51·11	54·57	28·86

An appreciable amount of sugar was broken down during the hydrolysis of the glucoside, the rhamnose being more easily destroyed than the glucose. The extent of this breakdown, which is shown in the low values obtained by both methods of analysis, is not so apparent in the polarimetric as in the copper reduction estimations, since, as the rotation of rhamnose (+ 9·0°) is small compared with that of glucose (+ 52·7°), a considerable loss of the pentose does not lower the combined rotation to a proportional extent. Willstätter found a similar discrepancy in the values obtained on estimating these sugars from keracyanin chloride and the flavone rutin by the same two methods.

SUMMARY.

1. The magenta anthocyanin, *antirrhinin*, has been isolated from the crimson flowers of *Antirrhinum majus* as a crystalline chloride.

2. Antirrhinin is shown to be a 3-rhamnoglucoside of cyanidin. It is probably identical with keracyanin, the pigment of the fruit of the cherry, but differs from sambucin from the elderberry.

3. The identification of antirrhinin as a cyanidin compound contradicts the theory of a simple relationship between this pigment and the ivory flavone apigenin, which is indicated by a study of the Mendelian factors for flower colour in *Antirrhinum*.

I should like to record my gratitude and thanks to the Hon. Mrs Wheldale Onslow for suggesting this piece of research, and for her help and advice; also to Sir F. G. Hopkins and Prof. R. Robinson for their interest in the work.

I also desire to thank the Department of Scientific and Industrial Research for a grant which has enabled me to complete these investigations.

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LXXXVI. NATURAL ANTHOCYANIN PIGMENTS.

II. THE MAGENTA FLOWER PIGMENT OF *PRIMULA POLYANTHUS.*

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(*Received May 1st, 1930.*)

INTRODUCTION.

In 1927 Karrer and Widmer [1927, 3] isolated and identified the anthocyanin pigments from three species of alpine *Primula*. They found that the flowers of *P. viscosa* and *P. integrifolia* both contained a pigment identical with malvin, a diglucoside of delphinidin-3′:5′-dimethyl ether, while from the flowers of *P. hirsuta* they isolated a diglucoside of a new *O*-trimethyldelphinidin, which they named hirsutidin. The pigment extracted from *P. integrifolia* was also shown to contain traces of some anthocyanin less highly methylated than malvin—a contamination to which these authors [1927, 1] had already drawn attention in the cases of other malvidin glucosides, such as oenin from grapes and myrtillin from bilberries.

Primulin, the anthocyanin pigment found in magenta and dark red flowers of *P. polyanthus*, is an interesting addition to this series. Like oenin, it consists chiefly of a 3-monoglucoside of malvidin, contaminated by traces of a lower methylated compound. Thus, although primulin furnishes the same aglucone as that found in two of Karrer's pigments, it differs from them in the nature and position of its glucosidal residue.

P. polyanthus is a horticultural hybrid probably derived from the cowslip and the common primrose. It is to be found in a large range of colours including various shades of yellow, magenta, crimson, orange and brick-red. Only magenta and crimson flowers were used for this investigation, as it is possible that a second anthocyanin is present in the orange and flame-coloured flowers.

The method of extraction and isolation was adapted from Anderson's [1923] modification of Willstätter and Zollinger's [1914] picrate method for the preparation of oenin chloride from purple grapes, in which 1 % aqueous hydrochloric acid instead of glacial acetic acid is used for the extraction of the pigment from the fresh flowers. Owing to the difficulty experienced in obtaining large quantities of material, only about 1 g. each of the pure crystalline hydrochlorides of the glucosidal and sugar-free pigments could be prepared.

The determination of the constitution of these pigments is based upon their appearance, reactions, solubilities, combustion analyses and absorption spectra, and upon the products obtained from the alkali fission and perhydrol oxidation of primulidin chloride.

The conclusion that primulin is not composed entirely of a monoglucoside of malvidin is based firstly upon the low values obtained on estimating the methoxy-content of primulidin chloride, and secondly upon the transitory purple colour obtained with its alcoholic solutions on addition of ferric chloride. This colour, which is also given by oenidin chloride, must be due to the presence of small amounts of delphinidin, or some methylated delphinidin which does not contain the syringic acid nucleus, or of cyanidin, since it is only obtained with pyrylium salts possessing free hydroxyl groups at positions 3′ and 4′.

Since oenin, the pigment of purple grapes, also consists of a monoglucoside of malvidin, together with traces of a less methylated compound, a comparison has been made between the reactions of primulin and oenin chlorides, and of the chlorides of primulidin, oenidin and pure synthetic malvidin. Oenin chloride was prepared from purple grapes by a method similar to that used for the isolation of primulin chloride. The specimen of malvidin chloride, together with samples of three synthetic methylated malvidin compounds, was very kindly sent me by Prof. R. Robinson, to whom I am greatly indebted.

A comparison between the appearance, solubility and reactions of primulin and oenin chlorides shows that they resemble each other in most respects, but differ in their "distribution number."

Primulidin chloride resembles pure malvidin chloride in its sodium carbonate reaction and in its sparing solubility in hydrochloric and sulphuric acids. In crystalline form, difficulty of recrystallisation and ferric chloride reaction it resembles oenidin chloride. The absorption spectra of these three pigments are almost identical.

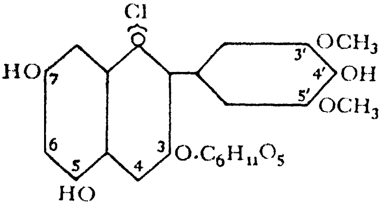
These anthocyanins and anthocyanidins were further compared by means of their colour reactions and stability in a series of buffered solutions [Robertson and Robinson, 1929]. Although primulin and oenin chlorides gave an almost similar range of colours, above p_H 6·8, those of primulin were slightly more blue. The chlorides of primulidin and oenidin differed in their behaviour slightly but definitely both from each other and from pure malvidin chloride. These comparisons further demonstrated the probability that, although both consist chiefly of malvidin monoglucoside, primulin and oenin are not identical, since they differ in the amount of contaminating pigment present.

On hydrolysis, primulin chloride yielded one molecule of primulidin chloride and one of glucose.

On addition of sodium carbonate, solutions of the glucosidal pigment gave a violet colour, while primulidin chloride gave a pure blue. This indicates that the glucose molecule is attached at position 3, thus preventing the production of the clear blue which is obtained with the sugar-free pigment which has a free hydroxyl group at position 3 as well as at 5, 7 and 4′ [Pratt and Robinson, 1925; Robertson and Robinson, 1927, 1928]. This position of the carbohydrate residue is further stressed by the fact that the colour given by primulin chloride on solution in a buffer of p_H 8·6 is identical with that given by the methylated malvidin compound synthesised by Bradley and Robinson [1928], in which the methyl group is at position 3.

Pure malvidin was first obtained as a crystalline chloride by Willstätter and Mieg [1914] from the wild mallow, in which it existed as the diglucoside malvin. Evidence that the methoxyl groups were in positions 3′ and 5′ was given later by Karrer and Widmer [1927, 1], who isolated syringic acid from the products of mild alkali fission, and decided to re-name the pigment *syringidin*. This structure was later confirmed by Bradley and Robinson's synthesis [1928] of a 3 : 5 : 7 : 4′-tetrahydroxy-3′ : 5′-dimethoxyflavylium chloride identical with malvidin chloride.

The constitution of 3-monoglucosidylmalvidin chloride, the chief constituent of primulin chloride, may therefore be represented by the formula below.



EXPERIMENTAL.

The extraction and purification of primulin chloride.

After the removal of their yellow centres and colourless corolla tubes, 600 g. of fresh red flowers of *Primula polyanthus* were placed in a large Kilner jar, 600 cc. aqueous 1 % hydrochloric acid added, the flowers pressed under the liquid and the air bubbles dispelled. The jar was loosely covered and left to stand for two days. The dark purple extract was then filtered. After grinding in a mortar, the residue was left to extract for two more days with a further quantity of acid (400 cc.).

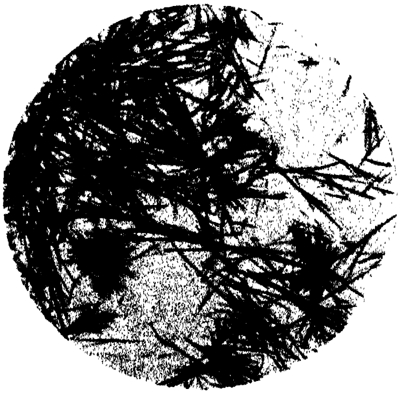


Fig. 1. Primulin picrate.

The first extract was warmed to 40°, and an equal volume of warm saturated picric acid added. The solution was kept at this temperature for a minute, and then allowed to cool to room temperature. A flocculent precipitate of microscopic scarlet-red needles soon began to separate out. (See Fig. 1.)

After standing in the ice-chest overnight, the picrate was filtered off on a small Büchner funnel, washed free from picric acid with ether, and dried in a vacuum desiccator. As the red-orange filtrate still contained a considerable amount of pigment, it was saturated with picric acid and used to precipitate the anthocyanin from the second crude extract by the same method as before. After the separation of this second yield of picrate, an attempt was made to recover further quantities of pigment by making the extract 10 % acid with hydrochloric acid. More pigment was thereby precipitated, together with picric acid, but the bulk still remained in the mother-liquor and could not be utilised.

The two yields of picrate, together weighing 4·5 g., were very finely ground in a mortar, dissolved in 150 cc. methyl alcohol containing 7·5 cc. hydrochloric acid and filtered. On addition of about a litre of ether, most of the pigment was thrown down in dark red-brown granules with a bronze lustre. The mother-liquor not only retained the picric acid, but also some of the pigment, but the latter could not be fully precipitated even by the addition of light petroleum.

The chloride was ground finely, and washed with ether, until the washings no longer gave a yellow colour on addition of alkali. The yield of crude amorphous glucoside was 3·3 g.

To obtain the pigment in crystalline form, it was dissolved in a minimum quantity of an equal mixture of methyl and ethyl alcohol (about 10 cc.) and one-third the volume of 10 % aqueous hydrochloric acid added. On standing in a loosely covered vessel, purple rod-shaped crystals of primulin chloride separated out either singly or in aggregates. These were filtered off, washed with a little cold 10 % hydrochloric acid, sucked as dry as possible and air-dried at room temperature. They formed a purplish red powder which marked paper with a purple streak.

Part of this yield was recrystallised for analysis, the strongly coloured filtrate being used for the preparation of the sugar-free pigment.

The glucosidal pigment, primulin chloride, $C_{23}H_{25}O_{12}Cl$.

Primulin chloride is very soluble in cold water, giving a deep red solution. On dilution it turns a plum colour, and then isomerises, with no precipitation of the colour-base.

The pigment has a maximum solubility in aqueous hydrochloric acid between concentrations of 1·0 % and 2·0 %, but is less soluble as the concentration increases. In all concentrations, the pigment is dissolved to a greater extent on heating.

Primulin is very soluble in methyl alcohol, and nearly as much so in ethyl alcohol. Both give intense crimson solutions, which, on dilution with more alcohol, at once turn mauve, and are then decolorised through isomerisation.

Primulin is insoluble in ethyl acetate; only slightly soluble in acetone when neutral, but more so on acidification.

The chief reactions of primulin chloride are given below.

Ferric chloride.	Plum colour in alcoholic solution, changing at once to a stable orange-red.
Sodium carbonate.	Violet colour in alcohol. Blue in thin layers, red-violet in thick layers.
Sodium hydroxide.	Clear blue.
Sodium acetate.	Plum-coloured solution in water, and in alcohol. A purple colour-base (insoluble in benzene) is sometimes precipitated from strong solutions.
Lead acetate.	Blue-violet precipitate.
Fehling's solution.	Reduced.
Potassium acetate.	Blue precipitate.
Perchloric acid.	Very soluble, no crystals of perchlorate formed.
Picric acid.	Soluble on warming, scarlet-red needles of picrate separate on standing. (See Fig. 1.)
7 % *sulphuric acid.*	Soluble even in the cold, no crystals of sulphate separate.

On drying *in vacuo* over phosphorus pentoxide at 105°, primulin chloride loses all of its water of crystallisation, but quickly regains half of it on standing in air.

On analysis, the following figures were obtained:

I.	0·2441 g. primulin chloride lost 0·0375 g. on drying	15·37 %
II.	0·4850 g. primulin chloride lost 0·0754 g. on drying	15·60 %
	Calculated for $C_{23}H_{25}O_{12}Cl, 5H_2O$	14·55 %
	Calculated for $C_{23}H_{25}O_{12}Cl, 5\frac{1}{2}H_2O$	15·85 %

Primulin chloride therefore appears to possess $5\frac{1}{2}$ molecules of water of crystallisation.

I.	4·386 mg. primulin chloride (dried) gave	8·390 mg. CO_2 and 1·91 mg. H_2O
	6·189 mg. ,, ,, ,,	1·682 mg. AgCl
II.	4·784 mg. ,, ,, ,,	9·100 mg. CO_2 and 2·08 mg. H_2O
	5·422 mg. ,, ,, ,,	1·480 mg. AgCl

The percentage compositions are therefore:

		C	H	Cl
By experiment.	Sample I	52·1	4·82	6·75
	Sample II	51·9	4·83	6·75
Calculated for $C_{23}H_{25}O_{12}Cl$ (oenin chloride)		52·2	4·74	6·74

The presence of methoxyl groups was demonstrated qualitatively by Zeisel's method.

The micro-analyses for carbon and hydrogen content of primulin chloride, and carbon, hydrogen and methoxyl content of the sugar-free pigment, were carried out by Dr Ing. Schoeller, of Berlin-Schmargendorf, and those for chlorine by A. Colwell, of this laboratory.

The colour reactions of primulin and oenin chlorides were compared in a range of buffered solutions, and found to be similar. Above p_H 6·8 the primulin chloride solutions were slightly more blue. The colours ranged from cherry-red at p_H 3·2, through rapidly fading violet-reds between p_H 3·6 and 5·6, and a fairly stable violet to violet-blue from p_H 6·8 to 8·0, to stable violet-blues (blue in thin layers) at higher p_H. In 10 % sodium hydroxide a very unstable blue was obtained. A violet colour-base was precipitated at all p_H values between 3·2 and 6·2.

Primulidin chloride, the sugar-free pigment, $C_{17}H_{15}O_7Cl$.

The crystalline sugar-free pigment, primulidin chloride, was obtained by hydrolysis of the glucoside with 20 % hydrochloric acid for 3 or 4 minutes. If heated too long, or if alcohol was present (as when the mother liquors from the crystallisation of primulin were hydrolysed), the pigment was partly amorphous.

Homogeneous crystals were prepared by dissolving the pure crystalline glucoside in hot 20 % hydrochloric acid and heating over a free flame for 4 minutes. The crystals began to appear after 2 minutes' boiling, and separated out almost quantitatively on cooling. Microscopically, they were pale brown or straw coloured rods, with blunt ends (see Fig. 2), their size depending upon the strength of acid used in the hydrolysis. The crystals were filtered, washed with a little cold 20 % hydrochloric acid and dried in air. They formed an almost black powder (pure malvidin chloride has a green reflex). The filtrate was later examined for the sugar residue.

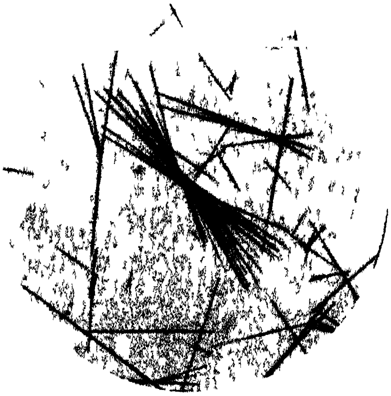


Fig. 2. Primulidin chloride.

Unlike pure malvidin chloride, primulidin chloride resembles oenidin chloride in being very difficult to recrystallise. Most attempts only gave amorphous forms, but small crystals were finally obtained by dissolving the pigment in water, and acidifying carefully until the solution contained 3 % of hydrochloric acid.

Primulidin chloride is slightly soluble in cold water, giving a red-brown solution after standing for half an hour. On heating, it is more soluble, but soon loses all its colour by isomerisation. The colour-base is precipitated from strong solutions.

The pigment is very soluble in methyl, ethyl and propyl alcohols, giving intense crimson solutions which, on dilution, turn purple and then isomerise. (Pure malvidin chloride is reprecipitated in small crystals from methyl alcoholic solutions.)

In amyl alcohol the pigment dissolves easily, giving a violet colour if neutral and cerise if acid. Like all anthocyanidins, on adding an excess of aqueous alkali, the pigment leaves the alcohol, turns blue, and dissolves in the water layer.

Ethyl acetate dissolves the pigment to a small extent; while acetone does so fairly easily, forming a red solution when acid, violet when neutral, and a clear blue if made alkaline with sodium bicarbonate.

Though practically insoluble in either water or concentrated hydrochloric acid, the pigment is slightly more soluble in concentrations of acid between 0·005 % and 1·5 %. Maximum solubility in the cold is found at a concentration of 0·03 %, while, when hot, 0·3 % acid has the greatest solvent power. This pigment therefore resembles malvidin chloride rather than oenidin chloride in respect of its solubility in water and aqueous acid.

The following reactions are given by primulidin chloride.

Ferric chloride.	Purple in alcoholic solution. Very unstable. (Malvidin chloride gives no colour reaction.)
Sodium carbonate.	Violet colour and then pure blue.
Sodium hydroxide.	Blue colour, more green than with carbonate.
Sodium acetate.	Red-blue dichroic solution in alcohol, purple in water.
Potassium acetate.	Blue colour, followed by precipitation of a colour base insoluble in benzene.
Lead acetate.	Dark blue precipitate of lead salt.
Fehling's solution.	Vigorously reduced.
Perchloric acid.	Soluble on standing, aggregates of badly formed hair-like crystals of perchlorate are formed.
7 % *sulphuric acid.*	Fairly soluble, especially on heating; almost complete precipitation of fine needles on cooling (as for malvidin chloride).
Picric acid.	Soluble on warming, fine red hair-like crystals in clusters separate on cooling.

The substance did not melt on heating to 300°.

On drying in a vacuum at 105° over phosphorus pentoxide, the air-dried crystals lose all their water of crystallisation.

The following values were obtained on analysis:

I. 0·0742 g. primulidin chloride lost 0·0048 g. on drying 6·47 %
II. 0·2963 g. primulidin chloride lost 0·0187 g. on drying 6·32 %
Calculated for $C_{17}H_{15}O_7Cl$, $1\frac{1}{2}H_2O$ 6·87 %

I.	4·705 mg. primulidin chloride (dried) gave	9·16 mg. CO_2 and 1·9 mg. H_2O
	4·260 mg. ,, ,, ,,	1·577 mg. AgCl
	3·502 mg. ,, ,, ,,	3·20 mg. AgI
II.	4·721 mg. ,, ,, ,,	9·63 mg. CO_2 and 1·75 mg. H_2O
	5·135 mg. ,, ,, ,,	1·863 mg. AgCl
	3·195 mg. ,, ,, ,,	3·22 mg. AgI

The percentage compositions are therefore:

		C	H	Cl	OCH_3
By experiment.	**Sample I**	55·3	4·28	9·10	14·76
	Sample II	55·6	4·12	8·96	13·40
Calculated for $C_{17}H_{15}O_7Cl$ (malvidin chloride)		55·9	4·11	9·68	16·90

These figures are in agreement with the dimethyl formula $C_{17}H_{15}O_7Cl$, $1\frac{1}{2}H_2O$. The low values obtained for the (OCH_3) content indicate the presence of traces of a lower methylated delphinidin.

The colour reactions and stability of solutions of primulidin chloride were compared with those of oenidin and pure synthetic malvidin chloride in a series of buffered solutions. The different amount of contamination in oenidin and primulidin was demonstrated by the slight differences in the reactions of

Table I.

	p_H	Time of observation	Oenidin chloride (O)	Primulidin chloride (P)	Malvidin chloride (M)
10 % HCl			Bluish red, stable	Same as O	Same as O
I	3·2	Initial	Bluish red, fading	,,	,,
		30 min.	Very faint pink	Colour gone	Colour gone
II	3·6	Initial	A little bluer than I, fading	Same as O	Same as O
		30 min.	Very faint pink	Colour gone	Colour gone
III and IV	4·4 and 5·0	Initial	Same as II, but more intense	Same as O	Same as O, a little less intense
		30 min.	Very faint pink	All colour gone	All colour gone
V	5·6	Initial	Plum colour, more intense	Same as O	Same as O
		3½ hr.	Very pale pink	,,	,,
VI	6·2	Initial	A little more violet and more intense than V	Even more intense than O	Same as O
		3½ hr.	Very pale mauve	Same as O	,,
		18 hr.	Very pale blue	,,	,,
VII	6·8	Initial	Much bluer, pure blue in thin layers	Slightly more mauve than O	Same as O
		3½ hr.	Very pale blue	Same as O	,,
		18 hr.	,,	,,	,,
VIII	7·4	Initial	Same as VII	Slightly more mauve than O	Same as O
		3½ hr.	Paler and greener	Same as O	,,
		18 hr.	Very pale blue	,,	,,
IX, X and XI	8·0–9·2	Initial	Pure blue	Slightly more intense than O	Same as O
		3½ hr.	Paler and greener	Same as O	,,
		18 hr.	Very pale blue	,,	,,
XII	9·8	Initial	Same as XI	Slightly more intense than O	Same as O
		3½ hr.	Paler and greener	Same as O	Pale green-blue
		18 hr.	Dirty blue-grey	Dirty grey-yellow	Dirty grey-yellow
XIII	10·4	Initial	Slightly greener than XII	Pure blue as for XII	Same as P
		20 min.			Green-blue
		3½ hr.	Paler and greener	Same as O	,,
		18 hr.	Yellow (blue tinge)	Yellow	Yellow
XIV	11·0	Initial	Blue, greenish tinge	Slightly greener than O	Same as P
		20 min.	Pale blue	Pale blue	Pale green
		3½ hr.	Grey-blue	Grey-mauve	,,
		18 hr.	Yellow	Yellow	Yellow
XV		Initial	Same as XIV	Same as XIV	Same as P
		20 min.	Pale blue	Dirty blue	Pale green
		3½ hr.	Grey-blue	Yellow	Clear yellowish green
		18 hr.	Yellow	,,	Yellow
XVI		Initial	Same as XIV	Same as XIV	Same as P
		20 min.	Pale blue	Dirty blue	Pale green
		3½ hr.	Grey-blue	Yellow	Yellow
		18 hr.	Yellow	,,	,,
XVII		Initial	Same as XIV	Same as XIV	Same as P
		20 min.	Pale blue	Grey blue	Pale green
		¾ hr.	Grey-blue	Brown	,,
		3½ hr.	Yellow	Yellow	Yellow

the three chlorides, which are shown in Table I. It will be seen that below p_H 11 the primulidin chloride solution is slightly more mauve and more intense than the corresponding solutions of oenidin and malvidin chloride. At p_H 11 both primulidin and malvidin chloride are more mauve than oenidin, and at higher p_H these two pigments are destroyed in a shorter period of time. The resemblance between primulidin chloride and malvidin chloride appeared to be slightly greater than that between oenidin chloride and malvidin chloride. The buffered solutions were prepared by the addition of the increasing amounts of standard 0·2 N sodium hydroxide to the British Drug Houses "Universal Buffer Solution." Up to p_H 11·0, the p_H could be calculated accurately.

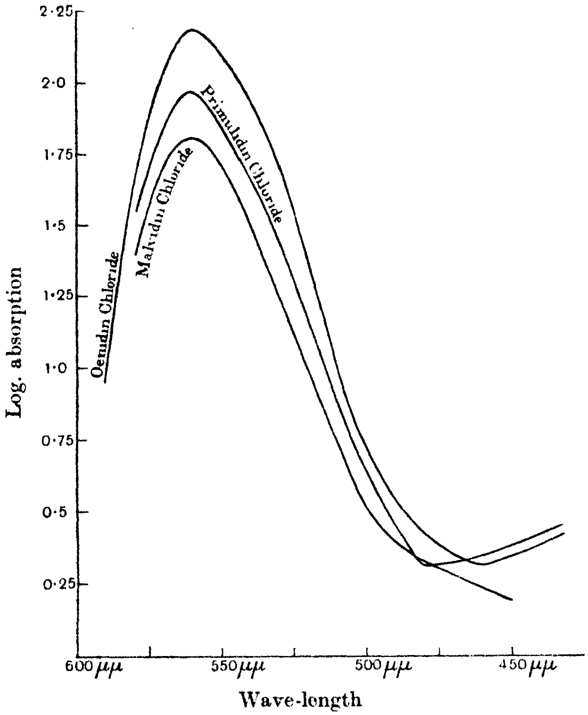


Fig. 3.

The absorption spectra of alcoholic hydrochloric acid solutions of oenidin, primulidin and malvidin chlorides were examined in the spectrophotometer, and the logarithmic curves were plotted to show the intensity of absorption of these three pigments within the visible spectrum. Each showed a band in the green portion, and a further one commencing in the violet and continuing into the ultra-violet region. The curves (see Fig. 3) were almost exactly alike, except at wave-lengths below 480μμ where the figures were unreliable, owing to the difficulty in taking accurate readings in the violet region. The maximum absorption in each case was at 560μμ. The pigment solutions used were of such a strength that the maximum log absorption values were at about 2·0. Owing to slight differences in these concentrations the curves obtained are parallel but not identical.

The breakdown of primulidin chloride by oxidation and alkaline hydrolysis.

After oxidation of primulidin chloride with hydrogen peroxide [Karrer and Widmer, 1927, 2], syringic acid was isolated from the decomposition products. About 0·2 g. of pigment was dissolved in 20 cc. hot water, and 15 cc. of 30 % hydrogen peroxide were added. After standing overnight, the brown residue was filtered off from the decolorised solution, which was then extracted several times with pure ether, and the ethereal solution washed free from peroxide by shaking twice with water. The ether was then evaporated off, and the pale brown residue was dissolved in a small amount of hot water, and decolorised by boiling with charcoal. On concentration of the solution in a vacuum desiccator, colourless needles separated. These were recrystallised twice, and identified as syringic acid by their melting point of 105° (uncorr.) and grey-green reaction with ferric chloride. The yield was very small.

A second sample of the pigment was treated for a few minutes with boiling 50 % potassium hydroxide, aerial oxidation being avoided. When hydrolysis was complete, the hot solution was poured cautiously into dilute sulphuric acid and extracted with ether. After treatment with sodium bicarbonate solution, through which carbon dioxide had been passed, it was necessary to extract repeatedly with ether to remove phloroglucinol. After evaporation of the ether, the pale yellow residue was taken up in water. The yield was very small, owing to the small amount of pigment available for the experiment, and the ease with which phloroglucinol is oxidised during the hydrolysis. It was possible, however, to identify it by the red colour obtained with a pine shaving and strong hydrochloric acid, by its orange-red diazobenzene compound, and by the precipitation of a brick-red phloroglucide on boiling with rhamnose and 20 % hydrochloric acid.

On acidification of the aqueous layer, extraction with ether, and evaporation, a pale brown residue was obtained, which appeared from its acid reaction and colour with ferric chloride, to be a mixture of gallic and syringic acids.

The sugar residue.

The identification of the carbohydrate portion of the primulin molecule was carried out by means of qualitative tests and quantitative estimations.

The "distribution number" for primulin chloride was ascertained by dissolving 0·005 g. in 25 cc. 5 % hydrochloric acid, and shaking with two successive amounts of amyl alcohol (25 cc.). The amount of pigment extracted in each case was measured colorimetrically by comparison with a standard made from 0·005 g. of pigment, dissolved in 1 cc. of methyl alcohol and made up to

25 cc. with amyl alcohol. The amyl alcohol was pyridine-free and contained a trace of hydrochloric acid to prevent isomerisation.

The mean value obtained for the distribution number of primulin chloride was 8·6. This is somewhat below the values usually found for monoglucosides (9·8 to 11·0), but is considerably above those for diglucosides, which are all below 2·0. (Oenin chloride has a distribution number of 10·4.)

About 0·1 g. of glucoside was hydrolysed with 10 cc. of 20 % hydrochloric acid. After cooling, the sugar-free pigment was filtered off, and a small amount of colour which still remained in solution was removed by shaking with amyl alcohol. The absence of pentose from the filtrate was shown by a negative orcinol test.

The remaining sugar solution was shaken with ether to remove any amyl alcohol remaining and neutralised with lead carbonate. After filtering, the excess lead was removed with hydrogen sulphide, and the latter was expelled from the filtered solution by heating on a water-bath.

The osazone was prepared from this neutral solution in the usual way. It resembled glucosazone in crystal form and in its melting point of 210° (uncorr.), which did not change after three recrystallisations from methyl alcohol.

The amount of glucose in the primulin molecule was estimated by determining the proportions of primulidin chloride and reducing sugar produced on hydrolysis of the glucoside.

A weighed quantity of primulin chloride (dried at 105° as before) was hydrolysed with 10 cc. of acid and left in the ice-chest until the mother-liquor was barely pigmented. The crystalline sugar-free pigment was collected on a weighed Pregl glass filter, washed with a small amount of 30 % hydrochloric acid, dried *in vacuo* at 105° over P_2O_5 and weighed.

The filtrate was extracted with amyl alcohol, shaken with pure ether to remove any remaining alcohol, neutralised with sodium hydroxide and made up to a definite volume. The reducing sugar present was estimated by Bertrand's method and polarimetrically.

I. 128·9 mg. primulin chloride (dry) gave 88·8 mg. primulidin chloride (dry)
128·9 mg. ,, ,, ,, reduced 80·0 mg. copper ≡ 39·82 mg. glucose
II. 84·1 mg. ,, ,, ,, gave 23·7 mg. glucose (25 cc., $l=2$ dm., $\alpha_D = +0\cdot10°$)
III. 53·9 mg. ,, , ,, gave 36·5 mg. primulidin chloride (dry)

The percentage yields are therefore:

	% $C_{17}H_{15}O_7Cl$	% $C_6H_{12}O_6$
By experiment I	68·8	30·9
II	—	28·2
III	67·7	—
Calculated for $C_{23}H_{25}O_{12}Cl$	69·4	34·1

The low sugar values obtained are due to the breakdown of an appreciable amount of glucose during the hydrolysis.

The analyses for the carbon, hydrogen and chlorine content of primulin chloride are also in agreement with the monoglucosidal formula. The polarimeter reading is correct for glucose, and not galactose.

The colour reactions of primulin, primulidin, oenin, oenidin and malvidin chlorides, and of three synthetic malvidin compounds methylated at 3, 5 and 7 respectively, were compared in buffered solutions of p_H 8. It was found that the chlorides of primulin, oenin and 3-*O*-methylmalvidin gave a similar violet colour (violet-blue in thin layers, and red-violet in thick layers). On the other hand, primulidin, oenidin and malvidin chlorides gave a similar pure blue, 5-*O*-methylmalvidin chloride gave a slightly less green-blue, and the 7-methyl compound gave a violet-blue (pure blue in thin layers, and blue-violet in thick layers), which was easily distinguishable from the violet colour given by the 3-substituted compounds. It is therefore reasonable to suppose that the glucose molecule in primulin is attached at position 3.

SUMMARY.

1. The magenta and dark red flowers of *Primula polyanthus* contain an anthocyanin pigment which has been named *primulin*.

2. By extraction of the pigment with dilute hydrochloric acid and purification through the picrate, pure crystalline primulin chloride has been prepared, and shown to consist of 3-monoglucosidylmalvidin chloride, together with traces of a lower methylated compound.

3. By a comparison of the properties and reactions of the chlorides of primulin and oenin and of the sugar-free pigments primulidin, oenidin and malvidin, it is shown that, although of nearly the same constitution, primulin and oenin are not identical.

4. Primulin furnishes the same aglucone as the pigments of *P. viscosa* and *P. integrifolia*, but differs from them in the nature and position of attachment of its glucosidal residue.

My thanks are due to the Hon. Mrs Wheldale Onslow for her help and advice, to Prof. R. Robinson for criticisms and suggestions and to Sir F. G. Hopkins for his interest in the work.

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LXXXVII. THE INFLUENCE OF INSULIN ON THE DISTRIBUTION OF GLYCOGEN IN NORMAL ANIMALS.

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SINCE the action of insulin began to be investigated, a puzzling discrepancy between its action on the diabetic and on the normal animal has attracted attention. In the diabetic animal it readily renews the store of glycogen in the depleted liver; on the other hand, the predominant result of the earlier and of much of the later work was to show that, when injected into the normal animal, if it measurably affected the glycogen in the liver or the muscles, it caused disappearance instead of additional accumulation [cf. Dudley and Marrian, 1923; Macleod and McCormick, 1923; Babkin, 1923; Nitzescu and Popescu-Inotesti, 1923; Brugsch, 1924; Gigon and Staub, 1923]. There was evidence, indeed, that insulin even in the normal animal might initially promote an increase of glycogen in the liver of the rabbit [Cori, Cori and Pucher, 1923], or of the whole mouse [Bissinger, Lesser and Zipf, 1923], though at a later stage of developed hypoglycaemia the glycogen became reduced below the original amount. Cori later found that insulin caused diminution of liver-glycogen in rabbits, guinea-pigs and mice when the proportion was initially high, but not if it was low. There was no room for doubt, however, that the glycogen stores of liver and muscles might in some species be seriously depleted by insulin with a concurrent great reduction of oxidative metabolism; and the problem remained of accounting for the disappearance of circulating glucose under such conditions.

Macleod [1926] and his school have interpreted such results as pointing to the conversion of glucose into some substance unrecognisable by the ordinary methods of estimating carbohydrates, when insulin acts on the normal animal. It was found in this laboratory, however, that when insulin acted under uncomplicated conditions on muscle, the glucose disappearing could be fully accounted for by glycogen storage and oxidation [Best, Dale, Hoet and Marks, 1926]. It was accordingly urged that the phenomena seen in the whole animal were due to complication by another action of insulin, namely, a depression of the new formation of carbohydrate, so that oxidative metabolism was concentrated on that pre-existing, with consequent depletion of reserves.

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Substantially similar views as to this effective transfer of oxidation to carbohydrate had been put forward by Laufberger and by Cori and Cori.

More recent evidence has suggested yet another factor in the total effect. Cori and Cori [1928] have shown that in the rat a suitable dose of insulin diminishes the store of glycogen in the liver and increases that in the muscles, while a small dose of adrenaline affects the distribution in the other direction, causing glycogen to leave the muscles as lactic acid, which the liver rebuilds into glycogen. Experiments by Marks and myself, which are being published elsewhere, have confirmed this effect of adrenaline on muscle-glycogen and brought to light a new discrepancy, since we failed to find enough lactic acid to account for the glycogen thus discharged. Since a hypoglycaemic dose of insulin provokes accelerated secretion of adrenaline, the bearing of this complication on the total effect of an insulin injection had to be considered, and gave promise of reconciliation between the rival conceptions above mentioned.

Meanwhile, however, Goldblatt [1929] had published the results of experiments, made chiefly on young, fasting, normal rabbits. He found that injection into such animals of a dose of insulin sufficient to cause a profound hypoglycaemia, without convulsions, produced a substantial accumulation of glycogen in the liver. The change shown in his experimental records was striking, especially in view of the fact that so many workers, under conditions but slightly different, had looked for such an effect and failed to find it, or had even observed its direct opposite. It proved to be easy, as I shall show, under the conditions of Goldblatt's experiments, to confirm his observation with regularity. He has based on it a theory that the action of insulin is simply to retard the conversion of liver-glycogen into glucose, with resulting accumulation of glycogen and hypoglycaemia.

Such a theory, attractive in its simplicity, might have been easy to accept at a very early stage of the investigations on insulin. Its acceptance to-day would appear to involve the rejection of a large body of evidence obtained by other workers, on a number of species and by various methods. It seemed desirable to examine further the relation of the fact observed by Goldblatt to many which appeared to conflict with it. With this object in view I have studied the effects of nonconvulsant doses of insulin in normal animals of several species.

EXPERIMENTAL.

Methods.

In all experiments convulsions were avoided, the animals being killed when preconvulsive flaccidity and inco-ordination had developed. The animals were killed either by a blow on the head or, in the case of mice and fowls, by decapitation. The thorax and abdomen were quickly opened and blood for sugar estimation was taken from the heart. Glucose was determined by the method of Hagedorn and Jensen. The liver was quickly removed, weighed and cut up into boiling 60 % potassium hydroxide, allowing 1 cc. per g. tissue. In the case of mice the livers were not weighed but directly dropped into tubes containing boiling potash and the weights determined by difference.

In young rabbits it is possible to remove quickly and cleanly the whole of the posterior thigh muscles, and such samples were invariably taken for muscle-glycogen determinations. The muscle was dropped immediately into boiling 60 % KOH. The anterior thigh and posterior leg muscles were removed for free glucose estimation, being immediately disintegrated in ice-cold absolute alcohol. In fowls a portion of the left pectoralis muscle was removed for glycogen estimation and in ferrets the procedure was to strip the leg and thigh for such samples.

Glycogen was determined by Pflüger's method, the glucose in the acid hydrolysate being estimated by the modified Bertrand method as described by Best, Hoet and Marks [1926]. Muscle-sugar was also determined according to the method used by these workers.

RESULTS.

1. *Experiments on young rabbits.*

The experimental conditions were closely similar to those of Goldblatt's experiments on insulin alone. In each experiment a batch of healthy young rabbits from one litter was taken and kept without food for 24 hours. In each case approximately equal numbers were taken for injection with insulin and as normal controls respectively. The ages of the litters varied from 6½ to 8 weeks, and the weights of individual rabbits in the whole series from 400 to 900 g. The average weight of 11 controls was 654 g. and of 11 rabbits receiving insulin 620 g. In a certain number of his experiments Goldblatt estimated the glycogen in the gastrocnemii of each rabbit as well as that of the liver. The number of such estimates on rabbits receiving insulin alone was hardly large enough, however, to justify the attribution of much significance to the relatively small deficit of muscle-glycogen observed, as compared with that in the few controls; and Goldblatt did not, in fact, lay stress upon it. I have estimated the glycogen in muscles as well as liver in all experiments on rabbits, and, to increase the chance of obtaining representative samples, have taken muscles from the whole of the posterior thigh in each case, as described under Methods. Table I shows the results on the 22 rabbits, estimates of liver-glycogen being available in all cases, and of muscle-glycogen in all except one of the controls, in which it was lost by accident.

There is clear confirmation of the increase of liver-glycogen observed by Goldblatt as the effect of insulin; the lowest figure in the insulin group is nearly 2½ times as great as the highest in the normal group, and the average insulin value more than 7 times as great as the normal. The effect on the muscle-glycogen is not so uniform. Of the 10 normal values, 2 are so low as to be within the general insulin range, and of the 11 insulin values 4 are high enough to be within general range of the normals. If we compare the simple averages of the two groups, that of the normal is practically twice as high as that of the insulin series. The figure in either case, however, is obviously subject to a rather wide range of error. It seemed worth while, nevertheless, to calculate for the glycogen from the liver and muscles the average total quantities per rabbit in the two groups, even though the figures could be regarded as only rough approximations.

Table I.

A. *Normal controls.*

Rabbit wt. g.	Liver wt. g.	Liver-glycogen %	Muscle-glycogen %	Muscle-sugar %	Blood-sugar %
476	13·96	0·24	0·19	0·100	0·131
480	16·08	0·25	0·18	0·099	0·163
462	14·62	0·60	0·21	0·117	0·150
464	17·28	0·20	0·30	0·127	0·141
852	19·33	0·28	(Lost)	0·115	0·152
582	15·18	0·08	0·66	0·103	0·110
800	20·30	0·12	0·42	0·127	0·106
690	20·06	0·57	0·08	0·097	0·132
904	23·97	0·42	0·12	0·103	0·122
804	24·41	0·33	0·30	0·124	0·156
676	17·55	0·22	0·27	0·112	0·138
Av. 654	18·43	0·30	0·27	0·111	0·136

B. *After insulin* (*0·5 unit*).

Rabbit wt. g.	Liver wt. g.	Liver-glycogen %	Muscle-glycogen %	Muscle-sugar %	Blood-sugar %
553	21·73	3·72	0·15	0·094	0·052
581	21·10	3·66	0·13	0·098	0·015
407	16·14	2·10	Trace	0·113	0·041
520	14·53	3·20	0·07	0·098	0·051
645	18·73	1·20	0·27	0·124	0·061
715	20·84	2·73	0·24	0·117	0·051
860	23·25	2·03	0·19	0·112	0·056
672	21·53	1·90	0·04	0·095	0·072
521	15·83	1·47	0·08	0·074	0·050
748	19·98	1·33	0·20	0·115	0·043
598	18·47	1·00	0·12	0·092	0·057
Av. 620	19·28	2·22	0·14	0·103	0·050

The livers were weighed in all cases, but a figure was required for estimating the weight of muscle from the total weight of a rabbit. Best, Hoet and Marks found that the muscles of a cat contributed approximately one-half of the total weight of the intact animal. Estimates were made on two young rabbits by the methods which they used.

Exp. Two young rabbits were kept fasting for 24 hours, weighed and then killed. The skin, the viscera of the abdomen and thorax and the brain were removed and weighed. The difference represented roughly the weight of muscles and bones. The stripped carcase was then boiled, and the bones were cleaned and weighed. The difference again gave the weight of the muscles. Table II shows the results.

Table II.

Rabbit wt. (g.)	809	797
Muscles and bones (g.)	504	482
Bones (g.)	106	79
Muscles (g.)	398	403
Wt. of muscles %	49	50

The muscle weight of the young rabbits, like that of the cat, can accordingly be taken to be about one-half the total body weight. Applying this factor, and the known average weight of the livers, we obtain figures for the average totals of liver- and muscle-glycogen in the two series, as shown in Table III.

Table III.

Averages	Normal	After insulin	Difference
Wt. of animal (g.)	654	620	—
,, muscles (g.)	327	310	—
,, liver (g.)	18·43	19·28	—
Liver-glycogen %	0·30	2·22	—
Muscle-glycogen %	0·27	0·14	—
Total liver-glycogen (g.)	0·055	0·428	+0·373
,, muscle-glycogen (g.)	0·882	0·434	−0·448
,, glycogen (g.)	0·937	0·862	−0·075

It will be seen that, on this calculation, the total loss of muscle-glycogen under insulin is actually slightly greater than the total gain of liver-glycogen. As already indicated, the figures for muscle-glycogen are too irregular and the method of calculation, involving a large and only approximate factor for weight of muscle, too rough to warrant the attribution of any significance to this difference. It is, however, justifiable to conclude that the gain of liver-glycogen, though much easier to detect with certainty, is not a more important item in the total change produced than the loss from the muscles.

Taken by itself, such a result would still be quite compatible with Goldblatt's suggestion as to the mechanism of insulin action. The normal level of the blood-glucose being maintained by liberation at an appropriate rate from the liver-glycogen, a stoppage of this process would lead the blood-sugar to fall, and the hypoglycaemia might in its turn prevent the muscle from replenishing the glycogen used in contractile metabolism. On the other hand, this combination of effects—the disappearance of glycogen from the muscles and its accumulation in the liver—is the direct reverse of that described by Cori and Cori as produced by insulin in the rat. Such a contrast clearly suggests the desirability of examining the effects in some other species.

2. *Experiments on chickens.*

All the chickens used were from a strain of the Plymouth Rock variety bred at the Institute's Farm Laboratories, Mill Hill, and were of a known and uniform age of about 3 months from hatching. The normal well-fed chicken, as others have recorded, has an apparent percentage of blood-glucose, as determined by reduction, about twice as great as that of the average mammal. It is stated that the true percentage of glucose is some 30 mg. lower than this reduction-value, which is partly due to uric acid, ergothioneine and other non-carbohydrate reducing substances. I have made no attempt to differentiate, recording the total reduction-values in all cases as glucose. It should be noted, however, that if an absolute correction of the amount above indicated were applicable to the figures for "blood-sugar" after insulin, a condition of very severe hypoglycaemia would in many cases be indicated, though no pronounced symptoms, and certainly nothing suggesting convulsions, occurred as the result.

The figures in Table IV, obtained from 3 chickens not deprived of food up to the time of killing, give an idea of the range of the "blood-sugar" and liver-glycogen in such normal birds.

Table IV. *Normally fed chickens.*

"Blood-sugar" %	Liver-glycogen %
0·199	3·64
0·200	2·77
0·188	2·4

It was found, however, that fasting for 24 hours sufficed to reduce the liver-glycogen to a very low proportion, so that in most cases it was below the range of trustworthy estimate by Pflüger's method, and had to be recorded as an unmeasured "trace." Chickens so prepared should accordingly offer an experimental material closely comparable to the young rabbits used in Goldblatt's experiments and my own. Birds, as others have observed, being very resistant to insulin, it was necessary to give it in relatively enormous doses to produce definite hypoglycaemic symptoms, 80 units per bird were injected in hourly doses of 20 units each, and the birds were killed when distinct symptoms of inco-ordination had appeared after the last dose. Table V shows the results on the glycogen content of the liver and muscles of these birds.

Table V. *Chickens after 24 hours' fasting.*

	Controls Percentages			Insulin Percentages	
Blood-sugar	Liver-glycogen	Muscle-glycogen	Blood-sugar	Liver-glycogen	Muscle-glycogen
0·199	Trace	0·50	0·052	Trace	0·51
0·175	,,	0·26	0·068	,,	0·32
0·200	,,	(Lost)	0·084	,,	0·61
0·188	,,	0·31	0·052	,,	0·85
0·199	0·26	0·43	0·052	,,	0·60
0·199	0·18	0·40	0·068	0·32	0·50
	Av.	0·38		Av.	0·56

It will be seen that, in complete contrast to the effect on young rabbits, there is here no sign of accumulation of glycogen in the liver as the result of the action of insulin. The glycogen of the muscles, on the other hand, shows a small, but probably significant increase under the action of insulin.

The conditions of absorption from the alimentary canal, as also of new formation of carbohydrate in the liver, might of course be different in the chicken and the rabbit, when both were subjected to fasting for 24 hours. It was, therefore, of interest to study the effects on the glycogen of the liver and of the muscles of giving known amounts of glucose by the mouth, with and without insulin, to chickens which had previously fasted for 24 hours. Several experiments of this kind were carried out, and, since the conditions in all were identical, and the results uniform in direction, the latter may conveniently be presented together, as in Table VI. It will be seen that 5 birds were kept fasting as controls, 9 were given glucose alone, in each case 3 g. as 20 cc. of a 15 % solution by tube into the crop, and another 9 the same treatment with glucose and 40 units of insulin hypodermically, 20 units being given at the same time as the glucose, and another 20 units an hour later. Two hours after the administration of the glucose the birds were killed for analysis.

Table VI.

	Fasting controls Percentages				3 g. glucose by mouth Percentages			3 g. glucose by mouth 40 units insulin hypodermically Percentages		
	Blood-glucose	Liver-glycogen	Muscle-glycogen		Blood-glucose	Liver-glycogen	Muscle-glycogen	Blood-glucose	Liver-glycogen	Muscle-glycogen
	0·184	0·18	0·73		0·226	1·95	0·38	0·251	0·08	0·81
	0·199	Trace	0·90		0·234	2·45	0·52	0·343	0·58	0·78
	0·190	0·22	0·49		0·213	2·28	0·73	0·164	0·29	1·08
	0·206	Trace	0·75		0·232	2·12	1·00	0·163	0·55	1·03
	0·206	,,	0·22		0·232	2·28	0·41	0·152	Trace	(Lost)
Av.	0·197		0·62		0·240	1·81	0·40	0·141	2·02	1·01
					0·243	1·90	0·62	0·102	Trace	0·62
					0·228	2·70	0·85	0·286	,,	0·48
					0·282	1·70	0·94	0·127	0·15	0·85
				Av.	0·237	2·02	0·65	0·192		0·83

The results, as regards the liver-glycogen are perfectly clear. Glucose alone by mouth, without extraneous insulin, causes a rapid replenishment of the glycogen store of the liver, depleted by fasting. This is accompanied by a moderate rise in the average level of the blood-sugar. When insulin is given in addition to the glucose, no definite hypoglycaemia occurs, some values being above, others below, the normal and the average being practically the same as in the controls. Nevertheless, except in one aberrant case, the insulin can be said to have prevented, or restricted to very small dimensions, the effect which the same dose of glucose by itself produced on the liver-glycogen. As usual, the figures for muscle-glycogen are more irregular, and the comparison of averages has a consequently less certain validity. They appear to indicate that, whereas glucose alone causes no perceptible change in the glycogen content of the muscles, insulin in addition to glucose causes some increase in the muscle-glycogen, whereas it prevents, as a rule, the accumulation in the liver which glucose alone causes.

Taking these results as they stand, and deferring for the moment any discussion of the processes concerned, we may note that the effects of insulin in the fowl, whether glucose is also given or not, resemble those described by Cori and Cori in the rat, and are in sharp contrast to those observed by Goldblatt and by myself in the rabbit.

3. *Experiments on young ferrets.*

These were chosen as being another type of animal, intermediate in size between the rabbit and the rat, purely carnivorous, and easily obtainable in litters of sufficient size. A few preliminary experiments showed that the liver of the ferret retains its glycogen store with great tenacity, from 2 to 4 % being present after 24 hours' fast, and that the species has a relatively high resistance to insulin, from 12 to 15 units being necessary to produce a recognisable hypoglycaemia in a young ferret. Even with such doses the only symptoms were muscular weakness and inco-ordination; no convulsions were seen.

Six young ferrets of the same litter, 7½ weeks old, were taken for an experiment. All were kept fasting for 24 hours. Three were then set aside as controls, and each of the other 3 received 12 units of insulin hypodermically. Three hours later all the treated animals were visibly flaccid and their movements inco-ordinate. All 6 were killed, and determinations made of blood-sugar, liver-glycogen and muscle-glycogen. The results are shown in Table VII.

Table VII.

	Controls Percentages			After insulin Percentages		
	Blood-sugar	Liver-glycogen	Muscle-glycogen	Blood-sugar	Liver-glycogen	Muscle-glycogen
	0·102	2·4	0·23	0·052	2·4	0·42
	0·092	3·8	0·31	0·052	1·9	0·45
	0·092	2·8	0·25	0·058	3·9	0·48
Av.	0·095	3·0	0·26	0·054	2·7	0·45

It will be seen that the insulin has produced a definite, though not extreme, hypoglycaemia. The liver-glycogen shows no significant change. The increase of muscle-glycogen, on the other hand, is consistent, and of significant dimensions.

4. *Experiments on mice.*

The effect of insulin on the distribution of glycogen between the liver and the muscles of the rat had been very thoroughly studied by Cori and Cori, and it seemed unnecessary to make further experiments on this animal. In mice, on the other hand, the early experiments of Dudley and Marrian, which first drew attention to the unexpected disappearance of glycogen under insulin action, were few in number. Lesser and his colleagues used a method which did not differentiate between liver and muscles. Further experiments on mice seemed, therefore, desirable and, in particular, it seemed important to discover whether insulin in any dose, in any relation to food, and at any stage of its action, could be shown to promote such storage of glycogen in the liver of the mouse as it does in that of the young rabbit. Under no conditions, however, has it been possible to demonstrate such an effect, even as a preliminary phase of the action. In some cases the muscle-glycogen was estimated by removing the viscera, the head and the skin, and working up the muscles and bones of the stripped carcase together.

Exp. Ten mice weighing 16 to 20 g. were kept without food for 12 hours. Five were used as controls and each of the other five received 0·05 unit of insulin subcutaneously. The mice were kept at the laboratory temperature, so that the effect of insulin was protracted. The first of the insulin mice showed symptoms of weakness after 70 minutes and was killed, with one of the controls. Thereafter as each injected mouse showed such symptoms, and before any sign of convulsions appeared, it was killed, together with a control mouse. In each case the liver was removed, and dropped into hot potash, and the carcase similarly treated. The 5 control livers were worked up together, and similarly the 5 insulin livers. Separate estimates were made of the carcase-glycogen and of the sugar in the blood taken from each animal at killing. The last mouse was killed about 90 minutes after the insulin was given. The results are shown in Table VIII.

Table VIII.

	Controls Percentages			After insulin Percentages		
	Blood-sugar	Liver-glycogen (Av.)	Carcase-glycogen	Blood-sugar	Liver-glycogen (Av.)	Carcase-glycogen
	0·168		0·10	(Lost)		0·12
	0·131		0·22	0·041		0·08
	(Lost)	2·1	0·15	0·026	None	0·08
	0·113		0·08	0·024		0·07
	0·140		0·12	0·034		0·07
Av.	0·138		0·15	0·031		0·08

Though the minute "glycogen" precipitate from the combined 5 insulin livers was duly collected and hydrolysed, it did not yield a titratable amount of glucose. The carcase-glycogen shows a probably significant reduction, but has not approached exhaustion.

Exp. Twenty mice were kept without food for 12 hours. Ten were kept as controls and each of the other 10 received 0·02 unit of insulin. All the mice were then placed in a thermostat at 37°, at which temperature the smaller dose of insulin caused symptoms of hypoglycaemia in 15 to 25 minutes. The insulin mice were, as before, killed as they reached the stage of preconvulsive symptoms, and the controls alternately with them. The blood samples and the livers of each group were pooled for analysis, so that the figures obtained and shown in Table IX are average percentages.

Table IX.

Controls		After insulin	
Blood-sugar	Liver-glycogen	Blood-sugar	Liver-glycogen
0·139	1·11	0·034	0·003

Again the liver is practically emptied of glycogen. Several larger series of mice were then taken, which had been feeding up to the time of starting the observation or, in one series, until 2 hours before that time. In each series 5 were taken as controls, and the remainder received 0·05 unit of insulin. All were kept at room temperature, the insulin mice being killed in batches of five at intervals varying from 5 minutes up to 1 hour after the injections. The glycogen percentage was estimated on the pooled livers of each such sample of 5 mice. The results are given in Table X.

Table X. *Liver-glycogen percentages.*

No. of mice	Controls	Time after injection of insulin			
		5 mins.	10 mins.	15 mins.	1 hr.
15	1·67	—	—	0·91	Trace
15	2·54	2·34	—	—	0·01
20	2·07	2·03	1·56	—	Trace

In these well-fed mice, also, there is no suggestion of increased storage of liver-glycogen at any stage. Five minutes after the injection, with the dose only partly absorbed, the liver-glycogen is not certainly diminished, but shows no sign of increase; in 10 minutes the decrease is becoming obvious; after an hour there is practically no glycogen left.

A further similar series of measurements was made on 20 mice which had been kept without food for 16 hours. After 15 had each received 0·02 unit of insulin, they were kept in the thermostat at 37° and killed in batches up to 25 minutes from the time of injection. Table XI shows the percentages of liver-glycogen.

Table XI.

	Controls	5 mins.	10 mins.	25 mins.
Liver-glycogen %	0·30	0·13	0·15	0·001

Again, the first perceptible effect of insulin is a diminution of the liver-glycogen, exhaustion being practically complete in 25 minutes under these conditions. These experiments are typical of a number of others in which the effect of insulin was tested on mice fully fed, or fasting for different periods, and killed for examination at varying intervals after the injection, from 5 minutes onwards. On a total of 310 mice, in no case was any increase of liver-glycogen detected, but always a decrease when a significant change had occurred.

Bissinger, Lesser and Zipf made their observations on mice which, after fasting for 18 hours, were given insulin with 50 mg. glucose. Under those conditions they observed an initial increase in the glycogen of the whole mouse, occurring at an earlier period than in similar mice receiving glucose alone. It seemed desirable to attempt to repeat this observation, and to estimate the glycogen separately in the liver and in the stripped carcase, consisting chiefly of muscle.

Exp. Eight mice were accordingly kept without food for 18 hours, after which 80 mg. of glucose were injected into each mouse, and 0·04 unit of insulin into each of four, the others being kept as controls. After 30 minutes all were killed and worked up in the usual manner, the livers in each group being pooled, and the carcases examined separately. Table XII shows the result.

Table XII.

Controls Percentages			After insulin Percentages		
Blood-sugar	Liver-glycogen (Av.)	Carcase-glycogen	Blood-sugar	Liver-glycogen (Av.)	Carcase-glycogen
0·104	0·15	0·099	0·103	0·08	0·11
0·160		0·091	0·170		0·13
0·106		0·055	0·160		0·11
—		0·051	0·101		0·11
	Av.	0·074		Av.	0·12

It will be seen that in this series the supply of glucose by injection has been adequate to prevent, in every case, a fall of the blood-sugar in the mice receiving insulin below the range observed in the controls. The muscles under these conditions show a gain of glycogen, small indeed, but with such regularity that it is probably significant. In the pooled livers, on the other hand, in spite of the maintained blood-sugar, the glycogen has fallen to a value corresponding to an average content of about one-half that in the controls, although the latter is itself already remarkably low.

Experiments on young rabbits with adrenaline.

In certain of his experiments on rabbits Goldblatt gave insulin and adrenaline. He evidently assumed that adrenaline by itself would lessen the glycogen in the liver, and that storage under the combined action, such as he observed, furnished evidence of the prepotency of insulin, keeping glycogen in the liver in spite of the stimulus of adrenaline to its discharge.

Earlier evidence had, indeed, given us clear warrant for the assumption that the action of adrenaline with regard to liver-glycogen is simply to promote its discharge as glucose, whether in the rabbit or other species. There was a body of evidence in favour of its promoting, under suitable conditions, a new formation of carbohydrate in the liver, as well as discharge of what was already stored; so that the balance of affect, whether gain or loss of glycogen, might differ according to the depleted or loaded state of the cells when the adrenaline was given.

Goldblatt's results with insulin on rabbits, however, which my own have closely confirmed, were so unlike those obtained on other species, either by myself or by other observers, as by Cori and Cori on rats, that it seemed desirable to make a few direct observations on the effects of adrenaline on the glycogen distribution in young rabbits, under the conditions of Goldblatt's and my own experiments with insulin.

Exp. Four young rabbits of the same litter, 8½ weeks old, were kept without food for 24 hours. Two were kept as controls, and the other two each received 0·75 mg. of adrenaline, given as three hypodermic injections of 0·25 mg. each at intervals of 20 minutes. Two hours after the first injection the animals were killed, and the liver and samples of the musculature worked up as usual. Table XIII shows that the well-marked hyperglycaemia was accompanied by loss of glycogen from the liver, and also, though in smaller degree, from the muscles.

Table XIII.

	Controls Percentages				After adrenaline Percentages		
Wt. of rabbit g.	Liver-glycogen	Muscle-glycogen	Blood-glucose	Wt. of rabbit g.	Liver-glycogen	Muscle-glycogen	Blood-glucose
1066	0·36	0·21	0·152	1058	0·03	0·11	0·268
1051	0·51	0·13	0·148	1021	0·02	0·06	0·320

Rabbits treated with such a relatively large dose of adrenaline are, of course, losing carbohydrate from the system through glycosuria. In Cori and Cori's experiments on rats, the doses of adrenaline which caused increase of liver-glycogen were such as produced no conspicuous hyperglycaemia. An attempt was made to produce similar conditions in young rabbits.

Exp. Two series were taken, 8 from 1 litter and 6 from another, 4 and 3 being kept as controls. The treatment of the others was exactly the same as in the former experiment, with the exception that the 4 larger rabbits of the first series received 0·15 to 0·17 mg. adrenaline each, and the 3 smaller ones of the second series received 0·1 mg. each. Table XIV shows the combined results.

Table XIV.

	Controls Percentages				After adrenaline Percentages			
Wt. of rabbit g.	Liver-glycogen	Muscle-glycogen	Blood-glucose	Wt. of rabbit g.	Liver-glycogen	Muscle-glycogen	Blood-glucose	Adrenaline dose mg.
1020	0·45	—	0·135	1015	1·87	—	0·168	0·17
686	0·46	0·55	0·141	164	0·92	0·36	0·215	0·15
576	0·46	0·44	0·145	798 (1)	0·46	0·22	0·252	0·16
722	(Lost)	0·40	0·138	886	1·60	0·19	0·155	0·17
500	0·10	0·46	0·148	492	1·94	0·16	0·163	0·10
535	0·13	0·40	0·120	509 (2)	0·61	0·20	0·148	0·10
495	0·10	0·34	—	558	1·40	0·13	0·163	0·10
Av.	0·28	0·43		Av.	1·26	0·21		

It will be seen that in every case except one, which is marked (1) in the table, the liver-glycogen is definitely higher than in any of the controls. In this instance it will be seen that the blood-glucose is raised to the level of a definite hyperglycaemia, only slightly below the range of those in Table XII.

In another instance, marked (2) in the table, the increase of liver-glycogen is relatively small in relation to the highest value of all the controls, but it is still some 5 or 6 times as abundant as in the controls of its own small group. In this case the blood-sugar is hardly above the normal range, and imperfect absorption of the injected adrenaline may, perhaps, be suspected. Even when these cases are included, the average liver-glycogen content of the 7 rabbits treated with adrenaline is 4 or 5 times as great as that in 6 available controls. The increase is almost as striking as that recorded by Goldblatt, and by myself, with insulin. The muscle-glycogen in the treated rabbits is again reduced to about one-half that in the controls.

Discussion.

If we look at the results here recorded as a whole, it is obvious that they cannot easily be interpreted on Goldblatt's supposition that inhibition of the discharge of glycogen from the liver is the primary and essential effect of insulin, to which all others are secondary. They show that the effect on which he based this suggestion, namely the increase of glycogen in the liver of the young rabbit, is an unusual rather than a constant and typical effect of insulin on the normal animal. In none of the other species here under experiment have I succeeded in detecting it at all: in the mouse, in which the most systematic effort was made to discover it as a temporary phase, I failed, even when sufficient glucose was given to prevent a fall of blood-sugar.

A comparison of the results of Tables I and XIV might suggest another possibility. Results immediately similar to those in Table XIV were obtained by the Coris in young rats also, with small doses of adrenaline; but in rats and in the species other than rabbits here examined insulin has been shown to produce a shift of the glycogen balance in the other direction, the muscles gaining and the liver losing glycogen. Is it possible, then, that the glycogen changes seen in the rabbit are due not to insulin at all, but to adrenaline secondarily secreted in response to it? There are many facts which forbid the assumption of so complete a paradox. Adrenaline, given in such a dose that we can safely regard it as acting in substantial excess, causes not storage but discharge of glycogen from the liver (cf. Table XIII). In an animal without a pancreas, and therefore without insulin, glycogen disappears from the liver, to be stored again when insulin is given.

These anomalies and apparent contradictions are familiar to anyone who has followed the investigations which, during the past 8 years, have been stimulated by the discovery of insulin. With the evidence before us of the readiness with which secretion of insulin is provoked by a rise and adrenaline by a fall of the blood-sugar, we may well hesitate before too confidently attributing the effects on glycogen distribution, following the injection of either of these hormones into a normal animal of any species, to the uncomplicated action of the one injected. To obtain a safe basis, however, for even a speculative estimate of the respective parts played by the two hormones in the production of any particular result, we must turn to the evidence provided by experiments of a kind enabling the action of each to be studied in isolation. For the effects on muscle-glycogen such evidence is relatively complete, and simple in its significance. Insulin by itself causes increase of the muscle-glycogen at the expense of circulating glucose [Best, Dale, Hoet and Marks, 1926]. Adrenaline by itself, as suggested by experiments of Cori and Cori on rats, and confirmed in experiments on the eviscerated spinal cat by Marks and myself, which are being separately published, causes discharge of glycogen from the muscle, but not as glucose. Accordingly, when injection of insulin causes hypoglycaemia accompanied by increase of muscle-glycogen, we may reasonably conclude that insulin itself is the predominant agent; when insulin hypoglycaemia, in the absence of convulsions, is accompanied by loss of muscle-glycogen, as in the young rabbit, we may reasonably suspect that secretion of adrenaline has caused glycogen to be discharged from the muscles faster than it is stored under the action of insulin.

The evidence as to the action of insulin and of adrenaline separately on the liver is not so easy to interpret. The restoration of glycogen to the diabetic liver by insulin naturally aroused the expectation that such an effect would be demonstrable on the isolated liver with artificial perfusion. With insulin alone attempts to detect this action have uniformly failed. It may properly be suggested that the maintenance of a physiological condition under artificial perfusion is peculiarly difficult in the case of the liver cells. This, however, will hardly account for the fact that a slow increase in the glycogen of a perfused liver may be retarded or converted into a decrease, but never accelerated, by addition of insulin to the blood [Bodo and Marks, 1928]. On the other hand, it is a matter of common experience that adrenaline accelerates the discharge of glycogen as glucose from a perfused liver. If the facts are valid, they seem to lead to the difficult conclusion that both insulin and adrenaline cause disappearance of glycogen from the liver, and that neither promotes its storage. There are, however, certain observations on record which, though they need critical repetition and extension, are at least suggestive.

v. Issekutz [1924, 1927] detected no direct action of insulin alone on the rate of breakdown of glycogen into sugar in the perfused livers of frogs and rabbits. Certain of his experiments, however, suggest an antagonism of insulin to the mobilising effect of adrenaline on liver-glycogen, and some even appear to show that, when insulin alone has failed to affect the output of glucose, adrenaline in addition may retard it. Cori and Cori have interpreted the storage of glycogen in the liver of the normal rat, following the injection of small doses of adrenaline, with concurrent loss from the muscles, as due to passage of lactic acid from the muscles, and its resynthesis to glycogen in the liver; but it is at least open to doubt whether this gain by the liver would occur in the absence of the pancreas. We are entitled, then, to consider the possibility that, when either insulin or adrenaline acts alone, or in great excess, it causes a diminution of liver-glycogen—either by increasing the rate of discharge in relation to that of new formation, as we can reasonably assume in the case of adrenaline, or by depressing the rate of new formation in relation to that of discharge, which is compatible with what is known of the action of insulin—but that some kind of balanced action of both is needed for the normal storage of glycogen in the liver. Such a view would certainly meet the case of the diabetic animal, where the liver, emptied of its glycogen, in spite of extravagant gluconeogenesis, under the unbalanced action of adrenaline[1], regains its normal store and rate of production when insulin is given in the dosage needed to restore the balance. In the normal young rabbit, again, we have found reason, from the effect of an insulin injection on the muscle-glycogen, to suspect that the action of insulin itself is complicated, to a greater extent than in other animals, by that of adrenaline secreted in response to it; and it is in this species alone, of those here examined, that storage of liver-glycogen has been found to follow injection of insulin. It may be doubted whether the whole of an effect following the injection of insulin into any normal animal can safely be attributed to insulin alone; but the storage of glycogen in the rabbit's liver seems to be more clearly under suspicion of secondary complication than most. The possibility that yet other hormones than adrenaline may play a part in effects following an injection of insulin must be kept in view, but there are no experimental data for its discussion.

SUMMARY.

1. The accumulation of glycogen in the liver of the young, fasting rabbit as the result of injecting insulin, described by Goldblatt, is confirmed.

2. Under such conditions the musculature loses at least as much glycogen as the liver gains.

3. Similar effects have not been observed in other species. Insulin causes no deposition of glycogen in the liver of the fasting chicken, but prevents its deposition there as the result of giving glucose, while causing its deposition in the muscles. In the ferret it causes formation of glycogen in the muscles and has little effect on that in the liver. In the mouse it causes rapid discharge of glycogen from the liver; when glucose is also given, glycogen is deposited in the muscles, but still disappears from the liver.

4. Injections of adrenaline too small to cause glycosuria produce, in the young, fasting rabbit, a change in glycogen distribution closely similar to that following injections of insulin.

5. It is argued that the effects of insulin on the normal animal are complicated by effects of adrenaline secreted in response to its action, especially in the rabbit.

It is a pleasure to express my gratitude to Dr Dale for his constant interest and criticism during the investigation.

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[1] The term "adrenaline" is used for convenience, to include also possible similar actions by sympathetic nerves to the liver.

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LXXXVIII. THE COMPARATIVE RATES OF ABSORPTION OF SUGARS FROM THE HUMAN INTESTINE.

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Hoppe-Seyler [1881] and Heidenhein [1894] first drew attention to the fact that simple physical laws could not explain the absorptive phenomena in the intestine. Höber [1899] compared the rates of absorption of several sugars and salts and found that dogs absorbed galactose slightly faster than glucose. Hédon [1900] compared the rates of absorption of glucose, fructose, galactose and arabinose from a loop of rabbit's gut. He found that from a 25 % solution arabinose was absorbed most quickly and then in order galactose, glucose and fructose. From isotonic solutions glucose was absorbed most rapidly. Working with "Vella" fistulae on a series of four dogs Nagano [1902] found that the monoses showed characteristic relative rates of absorption. Galactose was most rapidly absorbed and then in order glucose, fructose, mannose, xylose and arabinose. Solutions over 10 % were not used. Hewitt [1924] compared the rates of absorption of dilute solutions of glucose, fructose and galactose from loops of gut. In rabbits no very conclusive results were obtained, but in cats glucose was absorbed much more rapidly than fructose, while galactose occupied an intermediate position. All were absorbed at equal rates after killing the intestinal mucosa by hot liquids or sodium fluoride. Cori [1925], using standard rats, administered by stomach-tube a strong solution of the sugar to be tested and subsequently killed the animals after varying intervals, and the sugar still left in the whole of the alimentary tract was estimated. This method was an improvement on the older ones in that intact animals could be employed. Large numbers of rats moreover could be used and the results averaged. Cori placed the sugars in the same order as Nagano and found their relative rates (glucose = 100) to be: galactose 110, glucose 100, fructose 43, mannose 19, xylose 15, and arabinose 9. Macleod [1929] has recently criticised one or two points in Cori's technique, and Magee and Macleod [1929] have determined the rates of diffusion of sugars through isolated oxygenated loops of rabbits' intestine. No more definite conclusions were drawn than that hexoses were absorbed more rapidly than pentoses.

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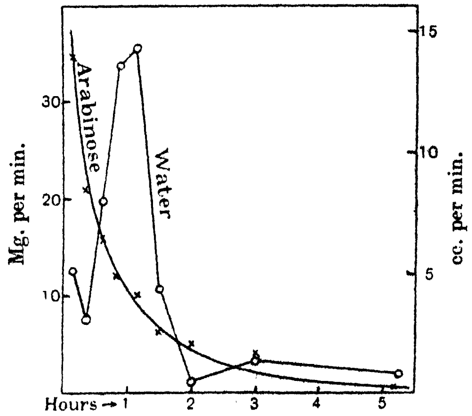
[1] Working with a part-time grant from the Medical Research Council.

Clearly none of the methods so far described is applicable to man, but we have devised a method by which the rates of absorption of rhamnose, arabinose and xylose have been compared. These three sugars are all excreted readily by the kidney and are only comparatively slowly destroyed in the tissues. We have therefore compared the rate and amount of each sugar excreted (*a*) when injected intravenously, (*b*) when taken by the mouth.

The sugars used were naturally occurring ones and were obtained from The British Drug Houses, Ltd. The xylose and arabinose each contained 0·2 % ash and 0·2 % moisture and were at least 98 % pure. The rhamnose contained 0·3 % ash and 15·8 % moisture which was allowed for in making the weighings. Dried, it melted at 121–125°. The estimations were all carried out by McCance's methods [1926, 1929]. Three normal adult men have been the subjects, H. age 23, wt. 64·5 kg.; H.L.S. age 25, wt. 57·2 kg.; R.A.M. age 30, wt. 66 kg. The procedure on each has been identical.



Subject H. Arabinose injected.

Fig. 1. Each point is plotted in the centre of the period during which that particular specimen of urine was secreted. It indicates in mg. per min. and cc. per min. the average rate of excretion of arabinose or water over that period of time.

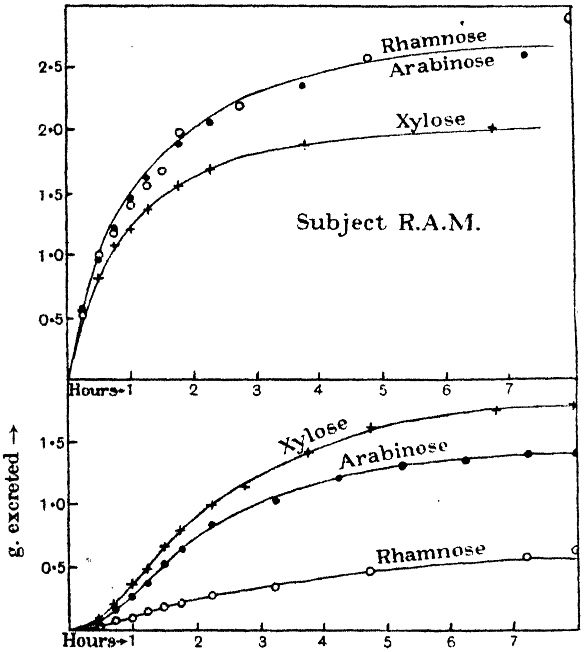
EXPERIMENTAL.

The effect of diuresis upon excretion.

Our experiments early established the fact that a moderate diuresis made little or no difference to the rate or amount of the sugar excreted. Fig. 1 illustrates this by comparing the rates of excretion of water and arabinose by subject H. The pentose was injected. Assuming the pentoses to be non-threshold bodies and that they are filtered off in the glomeruli we take this to mean that a diuresis of this degree does not involve the opening up of fresh glomerulo-tubular units to the circulating blood, but merely re-absorption of less water in the tubules [Rehberg, 1926]. Sometimes with small frequent samples of urine we have observed an increased output of sugar to accompany a small increase in urinary volume. This we have only observed when the rate of excretion was falling, and we regard it as being technical in origin. It is not always easy to empty the bladder completely at short intervals and a small amount of urine left behind would be voided with the next specimen and explain the experimental finding.

Injection experiments.

5 g. of the sugar to be tested were dissolved in 15 cc. of distilled water and sterilised at 100°. The whole of this solution was then injected slowly into the antecubital vein, the time taken for the actual injection being 4 mins. Zero time for the experiment was taken to be 2 mins. after the injection had begun. No tourniquet was applied. The bladder was emptied just before the injection and again every 15 mins. for 1¼ hours: later at extending intervals up to 7 or 8 hours when the experiment terminated. These experiments always began at about 10 a.m. and the subject had had a light breakfast. Small amounts of water were drunk at intervals. A moderate lunch of cheese and bread was taken about 1.30 p.m. at which water only was drunk. Fig. 2 (upper half) shows the detailed results from one subject (R.A.M.). The excretion curves of rhamnose and arabinose are almost identical. The rate is slower and the amount of xylose excreted less than that of the other two, but the shape of the curve is the same. In the case of subject H. the excretion curves of all three sugars were superimposable and are reproduced in Fig. 3. Differences in the rate and amounts of excretion (as in the case of xylose above)



o Rhamnose. • Arabinose. + Xylose.

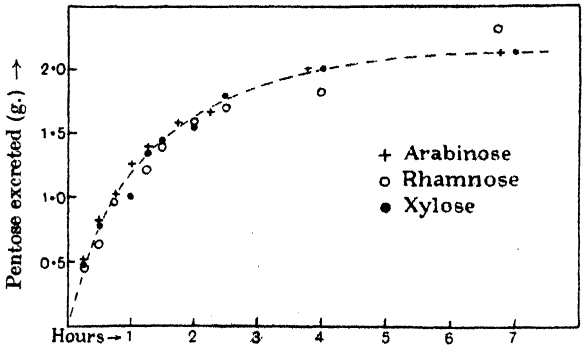
Fig. 2.

are presumably due to differences in the rate of utilisation by the tissues (see later).

There is no evidence that pentoses are stored as such in the animal body, and therefore these injected sugars can only be destroyed or excreted. The amount destroyed (or used) in any experiment can therefore be found by deducting the total amount excreted from the amount injected, *i.e.* 5 g.

Ingestion experiments.

Each subject took 5 g. of sugar by mouth and the excretion was again followed as before. After an overnight fast the subject emptied his bladder and drank 100 cc. water in which the sugar was dissolved. Urine was collected at the end of the first half hour and then for 1½ hours at 15 minute intervals. Thereafter collections were made at longer intervals up to 7 or 8 hours. Small amounts of water were taken from time to time. Fig. 2 (lower half) shows the actual results obtained from one subject (R.A.M.). These curves should be compared with those obtained from the same subject after the sugars had been injected. The initial delay in excretion shown by all three sugars is not due to the temporary delay of the solution in the stomach as will be apparent later. It should be noted that, whereas xylose is here excreted in greater amount than either of the others, less was excreted on injection.



Subject H. 5 g. injected intravenously.

Fig. 3.

Rhamnose is very poorly excreted when taken by the mouth, though readily after injection, which confirms injection experiments of Voit [1897]. On the other hand the relative amounts of these three sugars excreted by our subjects do not correspond to those of Von Jaksch [1899].

From these curves we obtain for each sugar the total amount excreted after ingestion. From the injection experiments we have obtained the characteristic ratio $\frac{\text{sugar used}}{\text{sugar excreted}}$ for each sugar. By applying it we obtain the amount used in these ingestion experiments. The amount used + the amount excreted = the amount absorbed. In this way we can calculate the amount of each sugar which has reached the tissues in the time of the experiment.

Results.

By applying this method we have obtained the following results which may be put into tabular form.

Table I.

Injection experiments.

	Total amount excreted after injection of 5 g.			Total amount used			Ratio $\frac{\text{sugar used}}{\text{sugar excreted}}$		
Subject	Rhamnose	Arabinose	Xylose	Rhamnose	Arabinose	Xylose	Rhamnose	Arabinose	Xylose
R.A.M.	2·8	2·65	2·1	2·2	2·35	2·9	0·79	0·9	1·38
H.L.S.	2·3	2·7	2·3	2·7	2·3	2·7	1·17	0·79	1·17
H.	2·3	2·2	2·2	2·7	2·8	2·8	1·17	1·27	1·27
Average	2·5	2·5	2·2	2·5	2·5	2·8	1·0	1·0	1·27

Ingestion experiments.

	Total excreted			Total used			Amount absorbed		
	Rhamnose	Arabinose	Xylose	Rhamnose	Arabinose	Xylose	Rhamnose	Arabinose	Xylose
R.A.M.	0·62	1·6	1·8	0·49	1·4	2·5	1·1	3·0	4·3
H.L.S.	0·40	1·2	1·65	0·47	0·95	1·9	0·87	2·15	3·55
H.	0·82	1·5	2·2	0·96	1·9	2·8	1·78	3·4	5·0
Average	0·60	1·4	1·9	0·6	1·4	2·4	1·2	2·8	4·3

The consistency of these results is very satisfactory. It will be noted that subject H. absorbed more of all the sugars than the others and H.L.S. less. R.A.M. absorbed intermediate amounts of each sugar. If the rate of absorption of rhamnose is taken to be 1 the comparative rates become rhamnose: arabinose : xylose, 1 : 2·33 : 3·6. We may note in passing that the very small amounts of rhamnose absorbed explain the high coefficients of utilisation found by many of the pioneers on this subject [Lindeman and May, 1896].

Experiments with rats.

We decided to repeat some of Cori's experiments on xylose and arabinose and extend them to rhamnose. The rats used were obtained from Vitamins, Ltd. and were all adult males of an inbred strain weighing between 230 and 300 g. each. Four rats were used for each sugar in two lots of two each and the procedure was as follows. 2 g. sugar were dissolved in a small quantity of distilled water and the volume was made up to 5 cc. 3½ ins. of a No. 3 flexible rubber catheter was attached to the nozzle of a 2 cc. "Record" syringe and the whole was filled with sugar solution up to the 2 cc. mark. A rat was then lightly anaesthetised with ether, the catheter passed quickly into the stomach and 2 cc. discharged from the syringe. In this way each rat received 0·8 g. After 3 hours the rats were killed by coal gas and the whole alimentary canal was at once dissected out and placed in a beaker, covered with a little water and boiled. The intestines were then cut up into small fragments with scissors and extracted repeatedly with boiling water. The washings were made up to 200 cc. and the sugar was estimated. The amount so found deducted from 0·8 g. gives the amount absorbed.

Note. Both Cori and Macleod have considered the possibility that the intestinal flora may utilise the sugars sufficiently fast to invalidate this method of obtaining the amount of sugar absorbed. Cori's controls show that the bacterial action is negligible.

Table II.

Sugar	Amount absorbed (g.)				Average (g.)	Comparative rates rhamnose = 1	
						Rats	Man
Rhamnose	0·04	0·20	0·07	0·15	0·12	1	1
Arabinose	0·23	0·30	0·23	0·25	0·25	2·1	2·33
Xylose	0·45	0·47	0·44	0·40	0·44	3·67	3·6

The relative rates in man have been added to the table for comparison and it is unnecessary to emphasise the agreement. The relative rates of absorption of arabinose and xylose found by Cori, when reduced to our scale become 2·1 : 3·5, results almost identical with our own.

There can be little doubt that the epithelium of the human intestine reacts to sugars in the same way as that of the rat. The relative rates of absorption of rhamnose, arabinose and xylose have been shown experimentally to be the same in both. We may assume with considerable confidence that the comparative rates of absorption of glucose, galactose and fructose found in rats will also hold in man. It is probable also that the same relative rates hold for the dog [Nagano, 1902], but our knowledge of this very fundamental relationship between sugars on the one hand and intestinal epithelium on the other is regrettably incomplete. The limited experiments on rabbits have not been consistent. We do no even know, therefore, whether this peculiar relationship holds throughout the mammalia, much less at what stage in evolutionary history it appeared.

The kinetics of sugar absorption.

1. So far we have dealt only with the total amounts of sugar excreted or absorbed. Fig. 4 now shows the varying rates of excretion of all three sugars following their ingestion by H.L.S. It is at once evident that the peak of excretion occurs about 1 hour after they have been swallowed, whatever the sugar taken and whatever percentage of that sugar is ultimately to be excreted. This we interpret to mean that these sugars are all absorbed best at the same level in the intestine and that this level is high up near the pylorus.

2. *The actual absorption curves.* It is evident that the curves of excretion shown in Figs. 2 and 4 in no way resemble the actual curves of absorption. Sugar continues to be excreted long after it has ceased to be absorbed because the amounts temporarily stored in the fluids of the body are only slowly utilised or excreted. The fact that excretion is not yet quite finished

8 or 9 hours after 5 g. have been injected is proof of this. The curves of absorption can however be constructed from the following considerations. The amount absorbed up to any given moment is equal to the sum of:

(1) the amount excreted up to that moment;
(2) the amount used up to that moment;
(3) the amount still in the "tissues" at that moment.

The first is obtained by direct estimation. The second is obtained as follows. Assuming that the rate of excretion and the rate of destruction (or utilisation) are both proportional to the amount in the body at any given time, then the ratio $\frac{\text{sugar used}}{\text{sugar excreted}}$ is a constant whatever the time. We have already made use of this ratio to calculate the total amount of each sugar used. It can be employed in the same way to calculate the amount used at any given time. The third, *i.e.* the amount in the tissues at any moment, is proportional to the rate of excretion at that moment and can be calculated from it. *

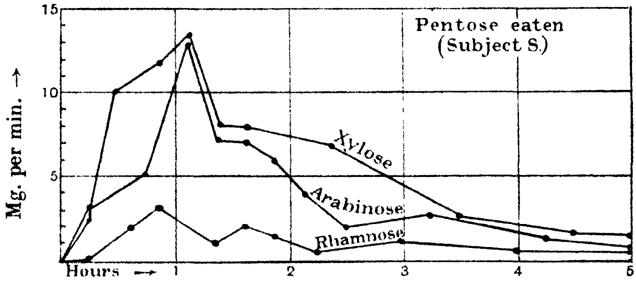
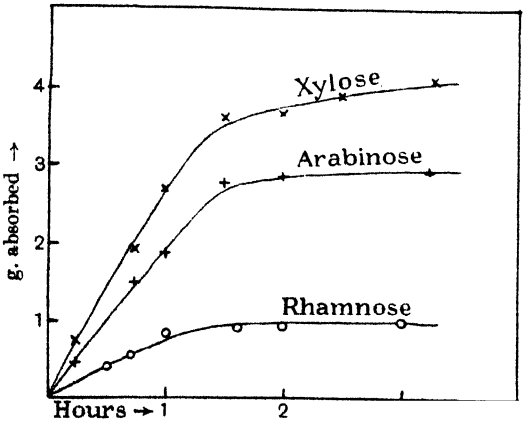


Fig. 4. Each point is plotted in the centre of the period during which that particular specimen of urine was secreted. It indicates in mg. per min. the average rate of excretion of sugar over that period of time.



Subject R.A.M.

Fig. 5.

The actual curves of absorption of subject R.A.M. are shown in Fig. 5, and should be contrasted with the curves of excretion of the same subject (Fig. 2, lower half). The exact determination of one of the points is given for the sake of clarity. The characteristic ratio $\frac{\text{sugar used}}{\text{sugar excreted}}$ for each sugar has been determined from the smoothed experimental curves and is for xylose 1·38 and for arabinose and rhamnose 0·82.

To calculate the amount of arabinose absorbed 1 hour after ingestion, proceed as follows:

(1) The amount excreted up to 1 hr. (by direct estimation) =0·25 g.
(2) The amount used up to 1 hr. =0·25 × 0·82 =0·19 g.
(3) The amount in the tissues.

The amount excreted between ¾ hr. and 1 hr. after ingestion =0·1 g. (by direct estimation); the amount similarly excreted between 1 and 1¼ hrs. =0·09 g. Assuming the rate of excretion to be constant for this half hour, the rate of excretion per hour at 1 hr. after ingestion =0·38 g. From the smoothed curve of excretion after injection this rate of excretion is found 2·05 hrs. from the start and at this time 1·95 g. have been excreted and therefore 1·95 × 0·82 = 1·6 have been used.

Therefore 5 − (1·95 + 1·6) = the amount in the tissues corresponding to a rate of excretion of 0·38 g. = 1·45.

Therefore the amount in the tissues 1 hr. after ingestion =1·45 g.

Total amount absorbed =1·905 g.

Great accuracy cannot be expected from these curves. In the first place they are based upon the assumption that the sugars distribute themselves equally throughout the body as soon as they are absorbed. This cannot be strictly true, but they certainly leave the blood stream and diffuse into the organs with great rapidity, for after 5 g. have been injected intravenously the curve of excretion is smooth and regular from the start. There is no excess of excretion in the first 10 mins. No serious error therefore, is involved in this assumption. Secondly, the determination of rates in this manner is not strictly accurate, particularly over parts of the curve where the rate is changing fast, *e.g.* between ½ and ¾ hour after ingestion. At the same time the samples of urine were collected over short periods (15 mins.), and the curves are extremely regular, so that there can be no doubt of the general truth of the results.

It is striking that the absorption of all these sugars should proceed rapidly (at a linear rate) for about 1½ hours and then almost or entirely cease when large amounts of arabinose and rhamnose presumably still remain in the gut. It is clear that absorption must commence as soon as the sugar is ingested and—as has already been pointed out—the upper part of the small intestine is the site of absorption. The lag in excretion is due to the time taken for the concentration in the tissues to rise.

Cori found that the rate of absorption in rats proceeded at a linear rate for 3 hours if enough sugar was given [Cori, Cori and Goltz, 1929; Pierce, Osgood and Polanski, 1929]. This Cori appears to consider a function of the epithelium. Cori's published results are very convincing and there can be little doubt that they are correct. Our own calculated absorption curves also show this tendency to a linear rate of absorption, at any rate at first, and it is to be recalled that we gave relatively very small amounts of sugar.

We would attribute the steady linear rate of absorption in Cori's cases (and in our own) not to a function of the epithelium, but to a slow steady emptying of the stomach. With rhamnose in our subjects absorption ceased while four-fifths of what was taken still remained in the intestine, and a steady linear rate of absorption could only have been obtained by a slow steady supply of fresh sugar to the absorbing areas in the upper part of the small intestine. The sudden cessation of absorption at $1\frac{1}{2}$ hours in our experiments we take to mean that after this time the stomach was empty and no fresh sugar was being passed down the intestine. It is significant that there was always sugar left in the stomach of Cori's rats.

Note. By making certain assumptions it is possible to calculate the rate of filtration through the glomeruli directly from the curves of sugar excretion following injection. Thus in Fig. 2 the rate of excretion after 2 hrs. = 0·38 g. per hr. Assuming this rate to be constant for 15 mins. this would lead to the excretion of $\frac{0\cdot 38}{4} = 0\cdot 095$ g.

1·95 g. have been excreted at this time. Therefore, $1\cdot 95 \times 0\cdot 82$ have been used = 1·6 and so $5 - (1\cdot 95 + 1\cdot 6)$ g. remain in the body = 1·45. Assuming the fluid content of the body to be 60 % of the weight, this subject contains about 40 litres, and assuming further the pentose not excreted to be uniformly distributed, the concentration of pentose in body fluids is 3·6 mg. per 100 cc., so that 2660 cc. must have been filtered off to give the 0·095 g. excreted, *i.e.* 178 cc. per minute.

Similar calculations from other curves give amounts of filtration varying between 100 and 170. The example given in detail is an exceptionally high one.

It is perhaps not quite legitimate to assume that the pentose sugars distribute themselves equally throughout the cells of the body, for Kozawa [1914] found that human red blood-corpuscles were impermeable to rhamnose although permeable to xylose and arabinose. Cori and Goltz [1925], however, have found that pentoses penetrate the tissues as readily and freely as hexoses, and the similarity of our excretion curves for the three sugars also indicates that this is true of the three pentoses. Accurate work on this subject would necessarily require estimations of pentose in the plasma.

These figures for glomerular filtration agree with those obtained by Rehberg [1926].

SUMMARY.

1. Rhamnose, arabinose and xylose are excreted equally readily when injected intravenously in man. Their curves of excretion are of identical shape and may all be superimposable.

2. The relative rates of absorption of rhamnose, arabinose, and xylose are the same both in rats and men. If the rate of absorption of rhamnose = 1, those of arabinose and xylose are 2·33 and 3·6 respectively.

3. These sugars are all absorbed at the same level in the intestine and this level is high up in the small intestine. Lower down little or no absorption takes place.

4. In normal man absorption of these sugars proceeds rapidly and at a linear rate for $1\frac{1}{2}$ hours and then almost ceases, even when large excess still remains in the intestine.

5. We may assume with reasonable confidence that the relative rates of absorption of glucose, galactose and fructose found in rats also hold in man, *i.e.* galactose is absorbed slightly faster than glucose, and the latter twice as fast as fructose.

We wish to express our thanks to Mr J. B. S. Haldane for some very valuable criticism.

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LXXXIX. STUDIES ON THE KINETICS OF HAEMOLYTIC SYSTEMS.

IV. A FURTHER STUDY OF THE SERIES OF RYVOSH.

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(*Received May 6th, 1930.*)

THE second paper of this series [Ponder, 1927] was concerned with an investigation, by quantitative methods outlined in the first paper of the series [Ponder, 1926], of the statement that the order of resistance of the red cells of various animals to saponin is the reverse of the order of resistance to hypotonic saline. The result of the study was to confirm the conclusion of Ryvosh [1907] that the following series holds for hypotonic saline, the most resistant species occurring first: man, guinea-pig, rat, rabbit, dog, pig, cat, ox, goat, sheep, and that the order of resistance to saponin is nearly the reverse. The same results have been more recently obtained by one of us [Yeager, 1928] and the facts thus seem to have been placed beyond question.

The explanation of the facts, however, is a very much more difficult matter. To make this clear, we shall tabulate the various suggestions which have been advanced.

(i) The resistance of red cells to saponin is stated to be determined by the quantity of cholesterol contained in them, and the lytic action of saponin to be due to its power of combining with this substance ([Yagi, 1911] and others).

(ii) Port [1922] has suggested that the phosphoric acid content of the cell is the essential factor in determining the resistance, a statement which is accepted by Orahovats [1926] and borne out by the recent analyses of Kay [1928].

(iii) Brinkman's theory regarding the resistance of cells to lysis by hypotonic saline implies that the resistance becomes greater as the cholesterol/lecithin ratio becomes greater [Brinkman, 1920], from which we may deduce that the resistance to saponin haemolysis becomes greater as this ratio decreases. This statement is incompatible with (i). Further, the conclusion of Meyer [1908] that red cells are the more resistant to saponin the higher the cholesterol/lecithin ratio, directly contradicts Brinkman's explanation.

(iv) In the second paper of this series we have suggested that saponin reacts with the cell proteins, and that the high negative correlation between phosphoric acid content and resistance to saponin is due to there being a correlation of + 0·79 between resistance and protein content (Abderhalden's figures [1895], not particularly reliable in this connection), and one of − 0·75 between phosphoric acid content and protein content.

(v) The resistance of red cells to digitonin haemolysis is stated to be parallel to the cholesterol content of the cell [Fabre and Simonnet, 1926]. This statement corresponds to (i), made for saponin.

(vi) Kofler and Lázár [1927] have shown that the order of resistance of various cells to saponin is by no means the same as the order to digitonin and to other lysins. A new series, in fact, arises with each lysin. This is certainly true so far as the bacterial haemolysins are concerned [Ponder and McLachlan, 1927], and one of us [Yeager, 1928] has shown that the resistance series for sodium taurocholate is quite different from that for saponin.

The difficulty associated with any of these suggestions as to the cell component with which the lysins combine is due to the obvious fact that if (vi) is correct, no one of the above suggestions can be used to explain the action of more than one haemolysin. For example, if (vi) is true, either (i) or (v), or both, must be wrong, for it is impossible for the action of two lysins to be determined by the quantity of the same component and yet for the two lysins to be uncorrelated with each other with respect to their activities. Similarly, if we explain the action of saponin in terms of (ii), and if (vi) be correct, the same explanation cannot be used to account for the action of the related lysin digitonin, or of any other lysin which gives rise to a resistance series other than that obtained with saponin.

THE EXTENSION OF THE RYVOSH SERIES.

The following table shows the resistances of the washed red cells of various mammals to saponin and to hypotonic saline. The measurements of resistance were carried out exactly as described in the second paper of this series, and might properly be added to Table I of that paper, as the suspensions contained the cells from an equal volume of blood.

Table I.

Animal	Saponin R	Saline %
Marmoset ...	0·57	0·40
Baboon ...	0·59	0·18
Lemur ...	0·64	0·42
Squirrel ...	0·90	0·27
Green monkey	0·90	0·43
Man	1·00	0·32
Opossum ...	1·10	0·35
Spider monkey	1·24	0·28
Sapajou „	1·42	0·38
Elephant ...	1·5	0·28
Macaque ...	1·55	0·22
Chimpanzee	1·70	0·28
Armadillo	2·10	0·31
Racoon ...	3·0	0·32
Kangaroo	3·5	0·32
Skunk ...	3·7	0·39
Wallaby ...	4·2	0·38
Black buck	6·0	0·46
Buffalo ...	7·0	0·42
Elk ...	8·0	0·42

As can be seen from a mere inspection of the figures, there is no obvious relation between the resistance to saponin and the resistance to hypotonic saline. The coefficient of correlation, in fact, is − 0·50, and that of rank correlation even lower, about – 0·3[1]. It thus appears that the conclusions of Ryvosh cannot be extended to the red cells of the mammalia in general, although they are correct for the animals included in the original series. We must consider how this can come about.

The essential point is that the ordinary theory of correlation applies only where large numbers are concerned and where random sampling is approached. Suppose, for the sake of argument, that the red cells of the various mammals are tabulated with respect to their resistance to saponin and to hypotonic saline, and that there is zero correlation between these two attributes, we shall, nevertheless, by a judicious choice of animals, be able to pick a small series which shows a high positive (or negative) correlation between the resistances referred to. For instance, from the 29 animals included in the above table and Table I of the previous paper, we can select the following series for resistance to saponin and to hypotonic saline respectively (least resistance first).

Hypotonic saline: sheep, goat, ox, cat, pig, dog, rabbit, rat, guinea-pig, man.

Saponin: rabbit, rat, man, guinea-pig, dog, pig, cat, goat, ox, sheep.

The rank correlation shown here is – 0·88 ± 0·071. Or we may select the following series (least resistance first).

Hypotonic saline: green monkey, lemur, marmoset, sapajou, racoon, man, armadillo, elephant, macaque.

Saponin: marmoset, lemur, green monkey, man, sapajou, elephant, macaque, armadillo, racoon.

The rank correlation shown here is + 0·70 ± 0·17, a result almost exactly opposite to that of the first series.

These two coefficients are different in sign, and both cannot possibly indicate the true state of affairs in the population they purport to describe; so it must, therefore, be possible for either to arise by chance from the particular population under consideration. The question is whether we can assess the chance. Where small numbers are concerned, there is no way of estimating the chance of a high correlation coefficient, positive or negative, arising from a large population showing zero correlation, for the usual tests, in which we compute the number of times the standard error is exceeded, fail altogether in cases such as this, where the true standard error of the coefficients is unknown. The standard errors of ± 0·07 and ± 0·17, attached to the correlation coefficients above, are, in fact, quite misleading, for they are calculated on the erroneous assumption that the numbers are large; if they were correct, the chances of the two coefficients arising by chance from a population showing zero correlation would be one in millions. The very fact that, out of a series of 29 animals, two such coefficients can be selected, shows that no significance can be attached to either as describing the true state of affairs in all the animals.

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[1] Calculation of coefficients of correlation, real or rank, from such a small number of cases as those dealt with in this paper is, of course, unsound statistical practice, and the coefficients have very little meaning. They are useful, nevertheless, for expressing a relation between two attributes, when the relation is not evident by simple inspection. As describing such a relation, even with small numbers, such coefficients are quite satisfactory: the danger in their use arises from the fact that we cannot assume them to be efficient statistics for a population including any cases additional to those from which they were obtained.

The series of Ryvosh accordingly appears to constitute a special case, involving the animals which, for economic reasons, are usually used in laboratories. This does not mean, of course, that there is actually zero correlation between resistance to saponin and resistance to hypotonic saline, for, were sufficiently extensive series examined, a significant negative correlation might still be found; on the existing evidence, however, we cannot say that such a correlation exists, and we may go even further and say that it is probable that it does not.

THE CELL COMPONENT DETERMINING RESISTANCE TO SAPONIN.

The fact that the cells of different animals vary some twenty times in resistance to saponin has suggested to many investigators that it may be possible to show that the varying quantity of some one substance accounts for the variation in resistance. The substances which have been suggested are (i) cholesterol, (ii) phosphates, and (iii) lecithin together with cholesterol, *i.e.* the cholesterol/lecithin ratio.

(i) The hypothesis that saponin produces lysis by combining with cholesterol rests upon several pieces of evidence: (*a*) that there is a high correlation between the cholesterol determinations of Abderhalden and resistance to saponin; (*b*) that digitonin, a substance allied to saponin, precipitates cholesterol quantitatively; (*c*) that Ransom [1901] and others have stated that the inhibitory action of serum on saponin haemolysis is due to the contained cholesterol uniting with the saponin and rendering it inert. We may place against these observations the following. (*a*) The analyses of Abderhalden give figures for total cholesterol, including esters which are not precipitated by saponin or digitonin, and are therefore rather misleading in this connection. (*b*) Kofler and Schrutka [1925] have measured the affinity of eleven kinds of saponin for cholesterol, and have shown that the combining power of saponin for this substance is by no means parallel to the lytic power. (*c*) Several observers have shown that the inhibitory action of serum on saponin haemolysis is due principally to its contained proteins [Ponder, 1923; Kennedy, 1925].

(ii) The hypothesis that saponin produces lysis by reacting with the phosphates (or with phosphate-containing substances) rests upon the fact that the coefficient of rank correlation between phosphoric acid content and resistance to saponin is very high (– 0·83, with Abderhalden's figures, or – 0·92 with Kay's figures). The appearance of the negative sign, however, completely eliminates the possibility of the lysin reacting with the source of the phosphoric acid, or of using the phosphoric acid content as a guide to the mode of action of the lysin, for the following reason. The whole of recent work indicates that haemolysis by saponin can be treated quantitatively on the assumption that, to produce lysis, a certain quantity of saponin, x, must be used up in combining with the cell membrane. The method of measuring resistance is such that the resistance of the cell increases as x increases, and hence as the substance attacked by the lysin increases. If the quantity of any substance in the cell membrane decreases as x increases, and if it is contended that the quantity of the substance determines lysis, the correlation coefficient has a negative sign; the meaning of the sign is that, whatever substance be attacked by the lysin, it is not this one.

(iii) The conflicting statements regarding the cholesterol/lecithin ratio have already been remarked upon.

The experiments hitherto done in this connection have generally failed to conform to one or all of three important conditions. (i) The usual methods for determining the resistance of cells to saponin really determine only the resistance of the most resistant cells, whereas chemical analyses determine the amount of substances contained in cells of average composition. Ideally, we should compare the content of the average cell with the resistance of the average cell population. (ii) The suspensions of the cells of various animals used in determining resistance must all contain the same number of cells, for, if the number is not the same, comparison of the results becomes almost impossible[1]. The quantity of a given substance obtainable from this number of cells can, however, easily be calculated from analyses on any known number of cells. (iii) It is important that resistance and chemical composition be determined for the cells of the same animal, and that analyses of the cells of one individual of the species shall not be compared with the resistance of the cells of another individual of the species, for the variation is great. It is not necessary, however, to employ large numbers of animals of the same species, for the resistance of the cells of one individual depends (by hypothesis) upon the amount of a given substance in its own cells, and certainly not, on any conceivable hypothesis, on the amount of the substance in the cells of other individuals.

With these points in mind, we have carried out a series of experiments in the following way. A set of dilutions of quillia saponin in 0·85 % sodium chloride, from 1 in 1000 to 1 in 560,000, was made in the usual way and stored in a refrigerator, being removed only when actually to be used. Blood was obtained from the animal (sheep, ox, horse, man, dog, pig, rat, rabbit and guinea-pig), the cells washed three times in 0·85 % NaCl and then made up to the original volume of the blood from which they were obtained. This suspension was used for the estimation of the following substances: (*a*) cholesterol (method of Myers and Wardell), (*b*) inorganic phosphates (Benedict and Theis), (*c*) total acid-soluble phosphates (Fiske and Subbarow) and (*d*) lecithin-phosphorus (Bloor, Benedict and Theis). The results were then reduced to quantities present per $0 \cdot 5 \times 10^6$ cells/mm.^{3}

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[1] The reason for this is that increasing the number of cells does not result in an equal increase in the quantity of saponin apparently used up. The increase in the latter figure is proportional to that in the former, but the proportionality constant is difficult to determine.

A small portion of the suspension was used for a cell count and a part of the suspension was afterwards diluted so as to give a suspension containing $0{\cdot}5 \times 10^6$ cells/mm.^{3}, which is of a convenient strength for the determination of resistance to saponin. The resistances were determined by finding the quantity of lysin necessary to produce 50 % haemolysis after 7 hours at 25°. All the experiments performed were completed within 2 weeks, thereby ensuring that the activity of the lysin remained unchanged.

The results may be described briefly as follows. (*a*) The coefficient of rank correlation between resistance to saponin and quantity of cholesterol is small and negative (– 0·6), and there is no evidence that the resistance increases with the quantity of contained free cholesterol. The results, in fact, lead to the same conclusions as those of Kofler and Schrutka. (*b*) The figures for total phosphates agree excellently with those of Kay. The correlation between the quantity of phosphates and resistance to saponin is again high but negative (– 0·75). Although the quantity of contained total phosphates may be a satisfactory guide to the resistance of the cell, the negative sign precludes the possibility of the haemolytic reaction being dependent, except indirectly, on the quantity of phosphates. (*c*) The coefficient correlating inorganic phosphates and resistance is also high but negative (– 0·80). (*d*) The cholesterol/lecithin ratio is not significantly correlated with resistance to hypotonic saline and therefore cannot be correlated positively or negatively in a significant manner with resistance to saponin (+ 0·45 to saline, – 0·30 to saponin). The coefficients of rank correlation above given are, of course, of no absolute value, but will serve as an incomplete description of the relation between the two attributes concerned.

THE HAEMOLYTIC ACTIVITY OF RELATED LYSINS.

The hypotheses hitherto discussed in this paper, *i.e.* those of Yagi, and Fabre and Simonnet regarding cholesterol, of Port regarding phosphates, and of Meyer and Brinkman regarding the cholesterol/lecithin ratio, have in common the fundamental assumption that the total quantity of some substance contained in the red cell membrane determines the resistance to lysis—that is, the minimal quantity of lysin required for complete lysis. If we accept this idea, and also suppose that chemically related haemolysins act upon the same component, it then follows that the cells of a series of animals arranged in the order of their resistance to one of the lysins are also of necessity arranged in order of their resistance to all the other lysins.

The only data on the subject of closely related lysins are those of Kofler and Lázár, and these agree with the result of observations on chemically unrelated lysins in showing that quite different resistance series arise when different lysins are used. The animals in Kofler and Lázár's experiments were sheep, ox, horse, man, dog, pig, white rat, rabbit, and guinea-pig, and Table II shows the order of resistance to different lysins, the figure 1 indicating the greatest resistance.

Table II.

	Saponin	Digitonin	Smilacin	Helleborein	Senegin	Gypsophila saponin
Sheep ...	1	9	8	3	2	1
Ox ...	2	8	9	4	5	3
Horse ...	3	4	7	7	9	2
Man ...	4	3	2	5	1	5
Dog ...	5	2	5	1	7	4
Pig ...	6	7	3	2	8	7
Rat ...	7	1	1	6	4	9
Rabbit ...	8	5	4	8	6	6
Guinea-pig	9	6	6	9	3	8
Coefficient of rank correlation		− 0·34	− 0·57	+ 0·52	+ 0·7	+ 0·88

As can be seen from inspection of the figures, and as is indicated by the coefficients of rank correlation between the resistance to saponin and to each of the other lysins, there is no uniform order of resistance.

In view of the importance of this conclusion, we have repeated the experiments using quillia saponin, digitonin and smilacin, and have determined the concentration required to produce 50 % haemolysis of a suspension containing $0{\cdot}5 \times 10^6$ cells/mm.^{3}. The orders of increasing resistance are shown in Table III, bracketed animals having as nearly the same resistance as can be determined experimentally; to Table III is also added the resistance series obtained with sodium taurocholate [Yeager, 1928] and with the lysins produced by *B. tetani* and by *B. histolyticus* [Ponder and McLachlan, 1927].

Table III.

Saponin	Digitonin	Smilacin	Sodium taurocholate	Lysin of *B. tetani*	Lysin of *B. histolyticus*
Rabbit	Sheep	Sheep	Guinea-pig	Rabbit	Sheep
Pig	Rabbit	Horse	Sheep	Ox	Ox
Rat	Ox	Ox	Dog	Horse	Rabbit
Man	Man	Pig	Rabbit	Man	Man
Guinea-pig	Pig	Dog	Rat	Sheep	Horse
	Cat	Rabbit	Ox		
Dog	Rat	Rat	Man		
Horse	Dog	Cat	Cat		
Cat	Guinea-pig	Guinea-pig	Pig		
Sheep		Man			
Ox	Horse				

The results in the first three columns confirm the conclusion of Kofler and Lázár, and show, as do the results in the last three columns, that the resistance series is different with each lysin. It should be observed that in the case of the first three lysins the order of resistance of the cells of the different animals is not identical with that found by those investigators; this is no doubt due to the fact that two samples of quillia saponin, smilacin and even of digitonin are often not entirely comparable[1], owing to their uncertain composition, and the same remark might well apply to two preparations of the bacterial lysins. The failure to obtain the same resistance series as Kofler and Lázár, however, leads to the conclusion that different orders are obtained not only with different lysins, but sometimes even with different samples of what is thought to be the same lysin.

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[1] Different specimens of digitonin, although stated to be chemically pure, have ,for example, different effects on the heart.

Since there is conclusive evidence that the cells of animals arranged in order of their resistance to one lysin are not also arranged in order of their resistance to other lysins, it follows that the haemolytic action of different lysins cannot be determined simply by the varying quantities of a single cell component, *e.g.* we cannot imagine the action of quillia saponin, digitonin and helleborein to be determined by the quantity of free cholesterol, cholesterol ester, phosphate, or any other substance contained in the cell membrane. It should be particularly observed that the nature of the cell component whose quantity is imagined to determine the resistance is of no importance, for the same argument would apply whatever its nature and whatever the affinity of the haemolysin may be for the substance, since varying affinities would not change the order of resistance.

If this conception is rejected, as it has to be, there remains the possibility that the resistance to a given haemolysin depends on the total quantity of a cell component, but that for each lysin a different component must be postulated. This explanation, more particularly, demands that there shall be as many components as there are resistance series, and it is possible to imagine such a number only by postulating that such compounds as "protein" are really made up of numerous different proteins, each one of which reacts specifically with each one of the many lysins. Speculations in this direction are fruitless at the present time.

The subject is much more profitably approached in the following way. In studies of the kinetics of haemolysis by simple haemolysins, the only postulate which has been found necessary is that the lysin acts on a cell component S and transforms a portion of it S_1, sufficient to make permeability to haemoglobin complete[1]. The resistance is measured in terms of this quantity S_1, or rather in terms of a quantity which is proportional to it, the quantity x of lysin used up in the process. This quantity x is directly obtainable from the figure for the smallest concentration (or the greatest dilution) required to produce a given amount of haemolysis, say 50 %, and, like the quantity S_1, has no necessary or even probable relation to the total quantity of S, except that S_1 cannot be greater than S.

It follows at once that there is no necessary or even probable relation between resistance and the total quantity of any cell component which may be mentioned. The suggestions of Port, Yagi, Meyer, Fabre and Simonnet, Brinkman and others really imply that S_1 always is equal to S, or is a simple function of S. The idea that such a relation exists is not only unsupported by evidence, but, as we have seen, leads to the conclusion that every haemolytic reaction is unique, in the sense that every lysin which presents a new resistance series must be imagined to act on a different substance.

When a lysin acts on S and transforms S_1 of it, the new compound is proportional in quantity to S_1, but its nature depends on (*a*) the nature of S_1 (which may be imagined to be a protein, different in different animals), and on (*b*) the nature of the lysin concerned in the transformation. The new substance S_2 which has resulted from the action of the lysin on S_1 is now a component of the membrane, just as S_1 was previously, and the permeability of the membrane depends upon (*a*) the quantity of S_2 and (*b*) its nature.

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[1] In the discussion which follows, it should be noted that the hypothesis advanced differs from all others in assuming that all lysins attack only the one component S in any given type of cell; the nature of S, however, differs from one type of cell to another.

To make clear the results which follow from those considerations, let us imagine four cases. (*a*) When destruction of semi-permeability is produced, the compound formed when saponin acts on human cells may be 20 micrograms in quantity, and its nature may be denoted by H-saponin, (*b*) the compound formed with ox cells may be 80 micrograms in quantity, and in nature O-saponin, (*c*) the compound formed with human cells and digitonin may be 80 micrograms in quantity and in nature H-digitonin and (*d*) the compound formed with ox cells and digitonin may be 20 micrograms in quantity, and in nature O-digitonin. Then the same result, *i.e.* destruction of semi-permeability is produced by 20 micrograms of H-saponin in human cells, but by 80 micrograms of H-digitonin in the same cells. This difference is easily understood when we remember that the compounds H-saponin and H-digitonin are different, and may, therefore, affect permeability to an unequal extent. But further, the same result is produced in ox cells by 80 micrograms of O-saponin and by 20 micrograms of O-digitonin. This difference is equally easily understood, for the two compounds are different. The figures for ox cells, furthermore, are not necessarily the same or in the same order as those for human cells, for not only is H not the same as O, but the human cell in which the compounds H-saponin and H-digitonin are formed is different in many other respects from the ox-cells, and the effect on permeability cannot, therefore, be expected to be the same in the two cases, even for the same lysin. *A fortiori*, it cannot be expected to be the same for two different lysins.

In short, the condition demanded by the existence of different resistance series is that the resistance of any cell to any lysin, whether denoted as a figure in micrograms, or as a relative resistance R, shall be a function both of the type of cell and of the nature of the lysin, *i.e.* $R = f$ (cell, lysin). The hypothesis proposed above meets this condition, for it states that $R = f(S_2)$ and $S_2 = f$ (cell, lysin). On the other hand, a hypothesis which assumes that lysis is determined by the total quantity of some one cell component, *e.g.* cholesterol, if extended to cover more than one lysin, fails to meet the required condition because it assumes that $R = f(S_1)$, the substance acted on, rather than $f(S)_2$, the substance formed as a result of the action, and, although S_1 is a function of the type of cell, it is not a function of the type of lysin. Furthermore, the hypothesis proposed above has the advantage that the kinetics of the haemolytic reaction postulated would be essentially similar for all lysins, as is in fact the case.

Since the lytic effect is supposed to depend, by this hypothesis, upon the formation of a compound whose nature and effect vary with (*a*) the type of cell protein and (*b*) the type of lysin, it is interesting to find (by calculation from Table III of Kofler and Lázár's paper) that the variation of resistance resulting from a variation in the lysin used is 3–4 times as great as that resulting from a variation in the type of cell. This is a result quite in accordance with expectation.

SUMMARY.

1. The conclusion of Ryvosh that resistance to hypotonic saline varies inversely with resistance to saponin in the red cells of mammals, although substantiated for the animals included in the original series, does not appear to hold when the cells of other mammals are studied, and it is doubtful whether this inverse relation is in any way a general rule.
2. Simultaneous chemical analyses and determinations of resistance to various lysins do not reveal any one substance whose total quantity can be said to determine the resistance of the cell.
3. *When the cells of various animals are arranged in order of their resistance to various lysins, a different order is found to be necessary for each lysin.* This *finding confirms the results of* Kofler and Lázár, and, although inexplicable *on the assumption that the resistance of the cell is determined by the quantity of some one substance in all cells*, is completely in accordance with the theory upon which studies of the kinetics of the haemolytic reaction are based.

We wish to acknowledge our indebtedness to the New York Zoological Society, and especially to Dr Charles Noback of the New York Zoological Gardens, for making it possible for us to obtain specimens of the blood of the various rare mammals mentioned in this paper.

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XC. THE DETERMINATION OF THE QUANTITY OF FREE WATER IN ERYTHROCYTES.

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The erythrocyte can be considered as a two-phase system, free water and dissolved salts occupying the continuous phase, while water bound with haemoglobin and lipoids constitutes the dispersed phase. The total water content of the cell has been shown by drying methods to be 70 % of the entire volume of the cell, but it is of some importance, in studying the partition relations existing between corpuscles and plasma, to determine accurately the relative proportions of free and bound water to be found in the cell. "Free water" may be defined as that proportion of the cell water that is osmotically transferable and "bound water" as that proportion that is not osmotically transferable under the same experimental conditions. *It will be seen that free water is then approximately equivalent to the "dispersion medium" of previous investigators, and bound water is a part of the "dispersed phase" (consisting of water bound with haemoglobin and lipoids). Two opposing conclusions have been reached by the method of determining changes in the* volume of corpuscles in saline solutions of varying osmotic pressures after prolonged centrifuging. Gough [1924] finds that the free water occupies 30–35 % of the cell volume, while Ege [1927] finds a value of 60 % for the free water. These results have been examined in the present investigation.

Gough suspends washed sheep cells in saline solutions varying in strength from 0·6 % to 4 %, spins them in a water centrifuge (*ca.* 2500 r.p.m.) for 3 hours, and then measures the height of the deposit of sedimented cells in the haematocrite tubes. Plotting the strength of solution against the cell volume reached after centrifuging, Gough finds: (*a*) that the curve for increasing volume of free water abstracted with increased concentration of NaCl assumes an asymptotic form, indicating that the maximum amount of free water can be abstracted by solutions of 3 % NaCl, and (*b*) that one must exclude a volume of two-thirds of the bulk of the corpuscle as not taking any part in the osmotic equilibrium. He thus obtains a value for the free water of 30–35 %, with the concentrations of NaCl he employs.

Ege, using the same method, concludes that the free water occupies 60 % of the cell volume, and believes that the source of error in Gough's determination is incomplete sedimentation of the corpuscles when suspended in hypertonic solutions. If the column of cells has not settled completely, owing to the presence of fluid in the interstices of the cells, the value for the diminished cell volume in hypertonic solutions does not represent the "true" volume, but a slightly higher value. This introduces a considerable error, according to Ege, in the amount of free water obtained from the cell[1]. Ege points out that centrifuging must be continued until the column of corpuscles is perfectly sedimented, *i.e.* until there is no interstitial fluid. The corpuscles have then attained their "true" volume as manifested by the column of blood taking on the translucent "appearance of laked blood" throughout its entire extent (Koeppe's criterion [1905]). If complete sedimentation takes place, as evidenced by the appearance of Koeppe's criterion, the value for the free water is found to be 60 % of the cell volume.

In the following experiments the question of complete sedimentation was considered and attention fixed on determining the maximum quantity of water that can be expressed from the cells (*i.e.* whether Gough's curve actually reached an asymptote in the concentration of NaCl used by him).

Exp. 1. Previous experimenters have used sheep cells freed from serum by washing three times in 0·85 % NaCl. These are then mixed thoroughly with equal volumes of NaCl solutions of varying concentrations and transferred to haematocrite tubes 10 cm. long. Similar preparations were made in this experiment, using NaCl solutions of from 3 to 20 %, and these were compared with preparations of whole unwashed blood (retaining the serum) mixed with equal quantities of the same NaCl solutions. The haematocrite tubes were spun in a high speed centrifuge (14,000 r.p.m.) for 10 minutes–1 hour. Constant volume was reached after about 12 minutes of spinning, but Koeppe's criterion was never observed even when the spinning was continued for 2 hours. The control in all cases was either washed cells (or whole blood) mixed with an equal volume of plasma. The determination is made by measuring the height of the column of sedimented cells in plasma and expressing this as percentage volume of the entire length of the column of liquid in the haematocrite tube. This is compared with the height of the sedimented column in the various saline solutions used, and the difference between readings in plasma and hypertonic saline, expressed as a percentage of the reading in plasma, gives the volume of free water transferred from the cell.

The average value for five experiments with washed cells gave 45 % free water, while the whole blood gave a value of 32 % for the free water. Similar discrepancies were noted for ox and rabbit cells, the cells washed in "isotonic" saline always giving higher values for the volume of free water. This would seem to be due to a factor hitherto neglected by previous investigators—that 0·85 % NaCl is not "isotonic" for most mammalian cells, and that many so-called "isotonic" solutions change the volume of the corpuscles by permitting an entrance of water[1]. In the following experiments, it was decided, as a consequence, to use only unwashed corpuscles, retaining the serum.

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[1] Gough's estimation of the height of column of sedimented cells is much less sensitive to errors than Ege believes, and the difference between the results of the two investigators cannot be explained in this way.

Exp. 2. The effect of time of spinning on the volume of cells was studied in an endeavour to determine the significance of the appearance of Koeppe's criterion.

Percentage volume of cells after spinning in haematocrite.

Time of spinning	5 mins.	10 mins.	12 mins.	14 mins.	16 mins.	20 mins.	30 mins.	1 hr.	2 hrs.
			Cells in 3 % NaCl.						
Ox	9·6	9·2	9·2	9·2	9·2	9·2	9·2	9·1	9·1
Sheep	15·4	15·1	15·0	15·0	15·0	15·0	14·9	14·9	14·8
Rabbit	12·3	11·8	11·8	11·8	11·8	11·8	11·8	11·6	11·6
Human	14·3	13·8	13·8	13·8	13·8	13·8	13·8	13·2	13·2
			Cells in 8 % NaCl.						
Ox	8·5	8·2	8·2	8·2	8·2	8·2	—	—	—
Sheep	14·3	13·9	13·9	13·9	13·9	—	—	—	—
Rabbit	12·3	11·7	11·7	11·7	11·6	11·6	—	—	—
Human	15·5	14·2	14·2	—	—	—	—	—	—

Constant volume was reached in some 10–12 minutes and prolonged centrifuging gave no appreciable decrease in cell volume. In no case did Koeppe's criterion appear[2], even with prolonged centrifuging with these hypertonic solutions. It would seem reasonable to expect its appearance after prolonged centrifuging at such high speed, according to Ege's results, but it was never observed, except under circumstances which will be explained later. It is important to observe the blank spaces in the table. These denote that cells in 8 % NaCl haemolysed after prolonged spinning, thus obscuring the readings, since the disintegrated cells were spread throughout the haematocrite tube and left no clear column of sedimented cells to be used as a basis for estimating cell volume.

Exp. 3. To determine the maximum quantity of water that can be expressed osmotically from the corpuscles, the cells were placed in concentrated saline solutions and spun until constant volume was reached. In many cases haemolysis took place and the cell volume could not be accurately measured on that account.

Tonicity	3 % NaCl	6 %	8 %	12 %	20 %
	Volume of free water osmotically transferred.				
Sheep	32·1	32·8	33·0	33·0	33·0
Rabbit	30·4	31·2	31·3	31·3	31·3
Human	31·3	33·1	33·2	33·2	33·2

According to theory, the amount of water leaving the cell varies with the concentration of the surrounding hypertonic fluid. Since we get no significant increase in the amount of water leaving the cell (*i.e.* since we have apparently reached the asymptote on Gough's curve relating cell volume to strength of

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[1] Unpublished data of Ponder and Saslow demonstrate these points quantitatively.

[2] It has been shown by experiments of Ponder and Saslow (now in press) that Koeppe's criterion is not coincident with the volume as determined by more accurate means such as the colorimetric method.

NaCl solution used) even with such strong solutions, we may conclude that the maximum quantity of free water has been transferred from the cell. In addition, it can easily be calculated that if the interior of the cell is assumed to consist of 100 unit volumes of a 1 % solution and if the surrounding fluid is a 4 % solution, sixty unit volumes of water would have to pass from the cell to the surrounding medium for osmotic equilibrium to be reached. If, then, we obtain less than this quantity even with more strongly hypertonic solutions, the reason is not that we cannot obtain any more water from the cell, but rather that there is no more water free to leave the cell. Solutions of KCl, sucrose and glucose were also used as suspension media but were not as successful in extracting the free water from the cells as were solutions of NaCl in similar concentrations. The experiments on sheep, ox, rabbit and human corpuscles accordingly confirmed Gough's conclusion that the free water occupies 30–35 % of the cell volume, provided haemolysis did not occur.

The appearance of haemolysis in suspensions in contact with strongly hypertonic solutions or subjected to prolonged spinning in the haematocrite complicated the experiments, but suggested the possible source of Ege's high value for the free water. When haemolysis occurs, the column of sedimented cells is decreased in height by the haemolysed corpuscles streaming up along the sides of the haematocrite tube. If this continues for any length of time, the entire tube becomes filled with the contents of the haemolysed cells and at the bottom of the tube is seen a slightly denser sediment of cells which appears somewhat translucent. The translucency, however, is not due to the close packing indicating Koeppe's criterion (which Ege describes as the "appearance of haemolysed cells"), but is due to an actual haemolysis taking place. Furthermore, the height of sedimented cells when haemolysed cannot represent the true cell volume as the cell contents have been liberated throughout the entire length of the haematocrite tube, and the cell volume is therefore very much less than the true volume. Such haemolysis with apparently smaller cell volumes and, consequently, larger amounts of free water, would seem to be the source of Ege's high value for the free water to be extracted from the cell[1].

SUMMARY.

Determination of the maximum change of volume of blood corpuscles in NaCl solutions of varying strengths gives a value of 30–35 % of the cell volume occupied by the free or osmotically transferable water contained in the corpuscle and 30–35 % for the value of the bound water, confirming Gough's figures.

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[1] Ege cites experiments by the method of determining depression of the freezing point by equal volumes of water and blood corpuscle press-juice in support of his high value for the free water. This method is not a reliable means of determining the relative proportions of free and bound water as it assumes two questionable points: (i) that the bound water is inactive in producing freezing-point lowering, and (ii) that the dissolved sucrose cannot be added to the bound water as well as to the free water.

Washing cells in "isotonic" solutions, prolonged centrifuging, the occurrence of haemolysis and the use of Koeppe's criterion as indication of true cell volume are to be noted as possible sources of error in Ege's determination of 60 % free water in the cell.

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XCI. THE USE OF THE BARCROFT APPARATUS FOR THE MEASUREMENT OF TISSUE RESPIRATION.

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(*Received May 1st, 1930.*)

The Barcroft apparatus was originally designed to measure the total amount of gas evolved or absorbed by a given process. Recently however it has been widely used for the study of tissue respiration and similar processes, in which not so much the total amount of gas absorbed as the *rate* of the process is to be determined. In order that the results should be significant in such cases, it is essential to ensure that the process is not being limited by purely physical factors depending upon the apparatus itself. Two such factors are likely to introduce errors, namely, inability of the oxygen to diffuse sufficiently rapidly from the gas into the liquid phase, and insufficient rate of absorption of the carbon dioxide, produced in the respiration, by the alkali present for that purpose. In spite of the considerable amount of work which has been carried out by means of the Barcroft apparatus on tissue systems, no data on the limitations of the apparatus from this point of view have yet been published. This question is dealt with in the present paper.

Details of apparatus.

The flasks of the apparatus were of the form illustrated in Fig. 1, this being the type used in this laboratory during the past seven years. The volume of the flask is 35 cc. and 3 cc. of liquid are used in all cases. The layer of liquid is about 1·5 mm. in thickness.

A considerable improvement in the reliability of the apparatus is effected by dispensing with the usual **T** taps, which were very liable either to leak or to become blocked, and substituting ordinary taps in the position shown in Fig. 1.

The liquid used in the manometers was paraffin coloured with Sudan III, which proved entirely satisfactory. Clove oil is to be avoided, as it is too viscous for use when rapid oxygen uptakes are being measured. The density of the paraffin used should of course be accurately determined before calibrating the apparatus, so as to avoid the necessity of recalibration in the event of accidental loss of the liquid. The area of cross-section of the bore of the manometer tubes was about 0·5 mm.²

The usual type of shaking mechanism was employed. This was normally adjusted to give a rate of shaking of 120 complete oscillations per minute, with a distance of travel of 2·5 cm., these conditions being those adopted in previous work. The shaking was not interrupted when readings were taken.

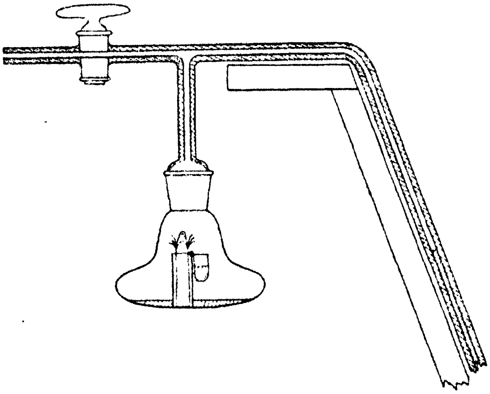

Fig. 1. Side view of apparatus. The drawing shows only one-half of the apparatus.

Absorption of carbon dioxide.

In using the Barcroft apparatus to measure the rate of oxygen uptake by tissue preparations, it is of course necessary that the effect of the CO_2 produced by the tissue shall be eliminated. For this purpose it has been customary to introduce about 0·3 cc. of a strong solution of NaOH or KOH into a small open tube sealed to the bottom of the flask, in order to absorb the CO_2. If any CO_2 accumulates in the gas phase, owing to incomplete absorption, the apparent oxygen uptake will be less than the true by a corresponding amount. If no alkali is added the movement corresponds to the difference between the oxygen absorbed and the CO_2 evolved, and this is made use of in determining the respiratory quotient (as described by Dixon and Elliott [1929]). In the course of the work just referred to we found that with the customary technique the rate of absorption of CO_2 was quite inadequate for experiments on rapidly respiring tissues. We therefore examined the efficiency of the absorption under various conditions.

For this purpose a Barcroft respirometer was set up containing in the flasks 1 mg. of sodium carbonate in the usual amount of liquid. In the right hand flask a small tube (as used by Keilin [1929]) containing 0·4 cc. of a strong solution of oxalic acid was suspended from the absorption tube by a small platinum hook as shown in Fig. 1. 40 % NaOH or KOH solution was placed in the absorption tube in the usual way. The apparatus was then shaken in the thermostat for 5 minutes, the taps closed, and the zero reading taken. After shaking for a further period to make certain that the reading did not change, the apparatus was gently tapped so that the tube containing the acid was upset into the carbonate solution and the CO_2 (about 200 mm.^{3}) thus liberated. The CO_2 was rapidly evolved, and readings were then taken for some time after the evolution had ceased in order to follow the rate of absorption by the alkali. Results obtained for NaOH at 14° and for KOH at 40° are shown in Fig. 2, curves A and B.

It is evident from these curves that the rate of absorption of CO_2 is quite insufficient, when the customary technique is used.

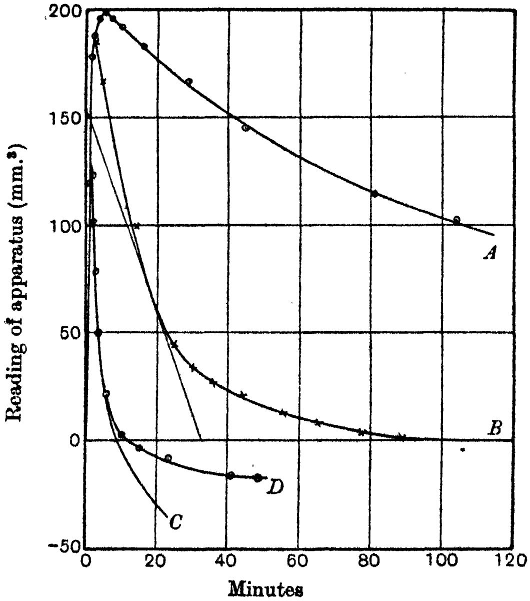

Fig. 2. Rate of absorption of CO_2 by various methods. Curve A; the absorbing agent was 40 % NaOH placed in the absorption tube; temperature 14°. (The curve for NaOH at 40° was slightly lower than this.) Curve B; 40 % KOH in the absorption tube; temperature 40°. Curves C and D; rolls of filter-paper were used in the right-hand flask only at 40°: Curve C; the paper was moistened with 40 % KOH; Curve D; 7 % KOH was used.

The tissue may be assumed to produce CO_2 at a constant rate. The rate of absorption on the other hand will depend upon the concentration of CO_2 in the gas phase. CO_2 will therefore accumulate in the gas phase until the rate of absorption becomes equal to the rate of production, and when this steady state is reached the movement of the manometer gives a true measure of the rate of oxygen uptake. Thus the error becomes smaller as the experiment progresses. It is possible to calculate the time required for attainment of the steady state and the magnitude of the error produced as follows.

Let x = the volume in mm.^{3} of CO_2 in the gas phase of the flask at any instant.

Let R = rate of evolution of CO_2 by the tissue in mm.^{3} per minute. R may be assumed to be constant.

The rate of absorption of CO_2 will be proportional to the amount of CO_2 in the gas phase. This will be the case no matter whether the rate is determined by the diffusion of the gas along the absorption tube or directly by the number of molecules striking the absorbing surface. Therefore we can write

$$\text{Rate of absorption} = cx \text{ mm.}^3 \text{ per minute},$$

c being a constant depending on the form of the apparatus, temperature, etc.

The rate at which CO_2 accumulates is equal to the difference between the rate of evolution and the rate of absorption. Therefore

$$\frac{dx}{dt} = R - cx.$$

On integrating, and determining the constant of integration by putting $x = 0$ when $t = 0$, we get

$$x = \frac{R}{c}(1 - e^{-ct}) \qquad \ldots\ldots(1).$$

At $t = \infty$, $x_\infty = \frac{R}{c}$.

We may obtain the time required for x to reach 99 % of its equilibrium value by putting $x = \frac{99}{100} x_\infty$.

Then $$\frac{99}{100}\frac{R}{c} = \frac{R}{c}(1 - e^{-ct})$$

whence $$e^{-ct} = 0{\cdot}01$$

$$-t_{99\%} = \frac{-2 \times 2{\cdot}3}{c}$$

$$t_{99\%} = \frac{4{\cdot}6}{c} \text{ minutes.}$$

We note that the time required for x to reach 99 % of its final value is independent of R, the rate of evolution of CO_2.

By drawing a tangent to curve B, Fig. 2, at any given point we obtain the value of cx, the rate of CO_2 absorption. *E.g.* when $x = 75$ mm.³ the rate of absorption $cx = \frac{153 \text{ mm.}^3}{34 \text{ mins.}}$, whence

$$c = \frac{153}{34 \times 75} = \frac{1}{16{\cdot}5}.$$

Therefore $t_{99\%} = 4{\cdot}6 \times 16{\cdot}5 = 76$ minutes.

Suppose CO_2 is evolved at the moderate rate of 300 mm.³ per hour. Then $R = 5$ mm.³ per minute, and $x_\infty = \frac{R}{c} = 5 \times 16{\cdot}5 = 82{\cdot}5$ mm.³

Using equation (1) we can plot x against t as in Fig. 3. We see that using this method of absorption, approximate equilibrium between CO_2 evolution and absorption would not be reached for 76 minutes, at which time 82 mm.³ of CO_2 would be present in the gas phase of the flask. Should the taps of the apparatus be closed after allowing say 10 minutes for the initial temperature equilibration, the curve shows that about 40 mm.³ of CO_2 are already in the flask, and therefore during the next 60 minutes a further 40 mm.³ of CO_2 will accumulate in the gas phase, so that the apparent oxygen uptake will be decreased by this amount.

Thus this method for CO_2 absorption is practically useless, and the following method, suggested to us by Dr Keilin, has been adopted.

A rectangle of filter-paper about 4 cm. by 2·5 cm. is rolled up fairly tightly into a cylinder, and tied with silk. The length of this roll should be about ¼ inch greater than that of the absorption tube in the flask. One end of the cylinder is then slit down by two cuts at right angles, and the ends spread out. This is then placed in the absorption tube as shown in Fig. 1, after lightly greasing the upper rim of the tube to prevent the alkali from creeping down the outside. The alkali solution is then run on to the paper.

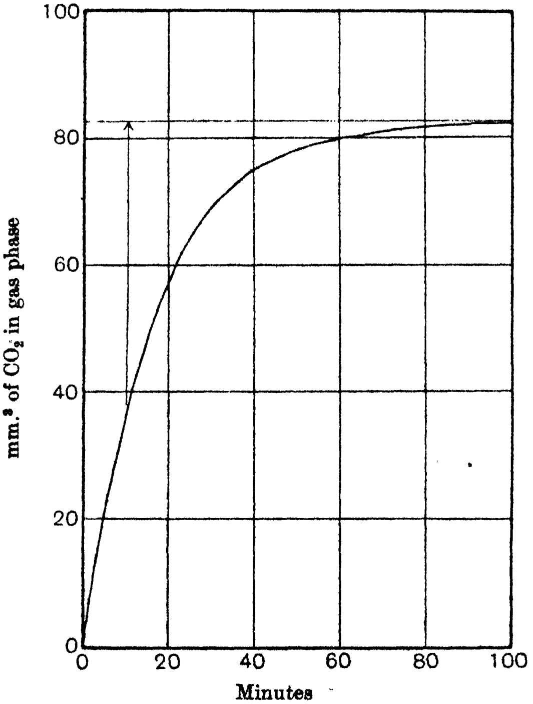

Fig. 3. The curve shows the accumulation of CO_2 in the gas phase with absorption conditions corresponding to curve B of Fig. 2, and with a CO_2 production of 300 mm.³ per hour. The length of the arrow represents the magnitude of the error produced when the taps are closed after 10 minutes.

The absorption of CO_2 is now very rapid, as is shown by curve C, Fig. 2. Using 40 % KOH at 40° it is evident however that a new factor has entered, for the amount of gas absorbed is greater than the amount of CO_2 present, and the pressure in the flask continues to fall after passing its original level. This was traced to the fact that under the action of the strong alkali the paper undergoes spontaneous oxidation with uptake of oxygen. With a weaker solution of alkali however the absorption of CO_2 is equally rapid, while the absorption of oxygen becomes nearly negligible. This is shown in curve D, Fig. 2, which was obtained using 7 % KOH at 40°. The inaccuracy due to the oxidation of the paper can be entirely obviated by having the alkaline paper in both flasks of the apparatus. In curves A and B, Fig. 4, 6 % KOH on paper was used at 14°, the paper being in the right hand flask only in curve A and in both flasks in curve B.

By calculation similar to that given above it is found from curve B, Fig. 4, that using this method of absorption only 11 minutes is required to reach approximate equilibrium, at which time 12 mm.^{3} of CO_2 are present in the flask if the rate of production of CO_2 is 300 mm.^{3} per hour as before. If the taps are closed after 5 minutes there will be 10 mm.^{3} already present, and the amount of CO_2 will increase by only 2 mm.^{3} which is entirely negligible.

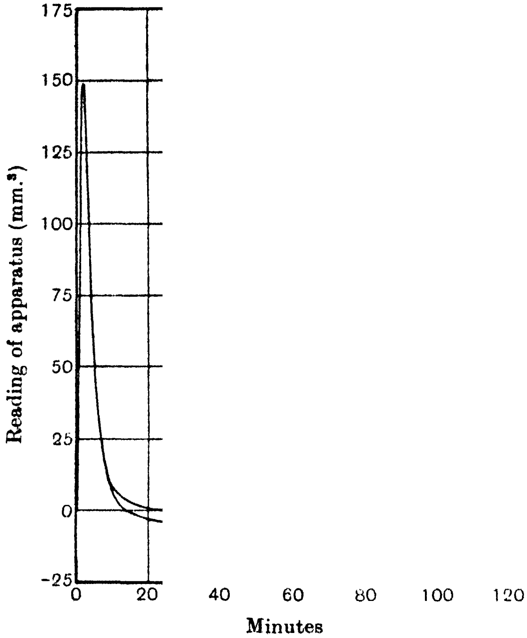

Fig. 4. This experiment was similar to that of Fig. 2. Curve A; 6 % KOH on filter-paper was placed in the right-hand flask only; Curve B; it was placed in both flasks of the apparatus. Temperature 14°.

Taking the high rate of CO_2 evolution of 1000 mm.^{3} per hour, the same calculation shows that the error when the taps are closed after 5 minutes would only be 5 mm.^{3}

This method is therefore entirely satisfactory.

Diffusion of oxygen as a limiting factor.

In order to measure the true respiration rate of cells immersed in a liquid it is necessary to ensure that the rate at which oxygen can diffuse into the liquid is rapid in comparison with its utilisation. (This applies also to the measurement of oxygen absorption by other systems.)

The importance of this was early appreciated when adapting the Barcroft apparatus for measuring rates of oxygen uptake, and Dixon and Tunnicliffe [1923] pointed out the importance of using flasks of a form exposing a large surface of liquid to the gas and giving efficient mixing of the liquid, and also of making sure in carrying out experiments that the rate of uptake is independent of the rate of shaking of the apparatus, this being an indication that the uptake is not being limited by diffusion effects. In spite of the considerable amount of work which has since been carried out with the apparatus, no quantitative data have yet been published on the rates of uptake which the apparatus is capable of measuring without errors due to diffusion effects.

The essential condition which must be fulfilled if the experiment is to be significant is that the concentration of dissolved oxygen must not fall so low that its rate of utilisation by the respiring cells (or other system undergoing oxidation) falls appreciably below that which would be obtained if the pressure of oxygen in the liquid were identical with that in the gas.

In order to make this clear let us consider what takes place when the amount of respiring cells, *e.g.* yeast, present in the 3 cc. of liquid in the flask of the apparatus is gradually increased. With very small amounts of yeast the rate of oxygen uptake will be small, and hence the pressure of oxygen dissolved in the liquid will be only slightly below that in the gas phase; the observed rate of uptake will be proportional to the amount of yeast present, and will be independent of the rate at which the apparatus is shaken (*i.e.* of the degree of mixing of the liquid). As the amount of yeast added is increased the rate of uptake is proportionately increased and the tension of dissolved oxygen will fall lower; but so long as it does not fall below the point at which the respiration rate of the yeast depends upon the oxygen pressure the rate of uptake is still a true measure of the respiration, and remains proportional to the amount of yeast and independent of the rate of shaking. A point is ultimately reached however at which the oxygen pressure in the liquid falls so low that the actual respiration rate of the yeast is cut down, when further additions of yeast have much less effect, and since the rate of uptake is now to some extent determined by the rate at which the oxygen can get to the yeast, it begins to be dependent upon the rate of shaking. Finally with large amounts of yeast the rate of uptake reaches a limiting value which is practically independent of the amount of yeast, and is entirely dependent upon the rate of shaking.

Owing to the complicated mixing conditions a quantitative mathematical treatment is hardly practicable, but we shall give here a number of experiments illustrating the effects met with and the capabilities of the apparatus.

The actual results obtained with yeast are illustrated in Fig. 5, which shows the relation between the observed velocity of oxygen uptake and the amount of yeast taken at two rates of shaking. (In all these experiments large "KOH-papers" were used, capable of absorbing the CO_2 produced by large amounts of yeast.) It will be seen that up to the point *X* on the curves the apparatus measures the true respiration rate of the yeast, since the velocity of uptake remains proportional to the amount of yeast taken up to this point; also the rate of uptake observed is the same at both rates of shaking. Beyond this point the velocity becomes limited by diffusion, and practically independent of the amount of yeast, at the slower rate of shaking. At the higher rate of shaking however the respiration rate can be followed up to considerably higher values before diffusion limits the process. It appears therefore that with yeast the apparatus is capable of accurately measuring uptakes up to about 1000 mm.^{3} per hour at the normal rate of shaking (120 per minute), at 15° and with the flasks filled with air. Correspondingly higher velocities can be measured at higher temperatures or with the flasks filled with pure oxygen instead of air.

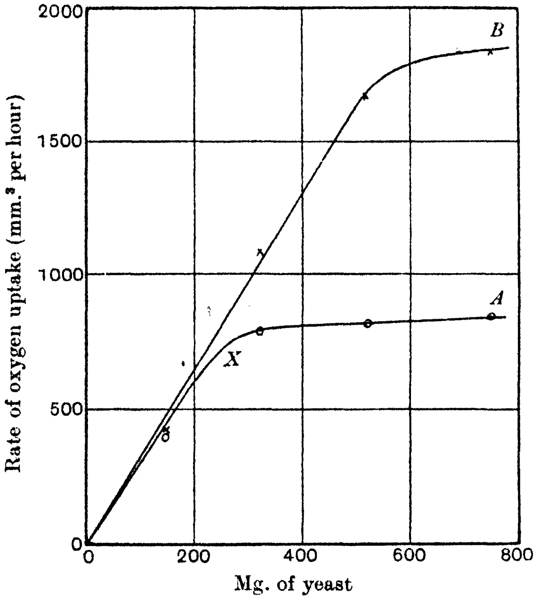

Fig. 5. Velocities of oxygen uptake observed with varying amounts of yeast. A suspension of 5 g. baker's yeast in 20 cc. phosphate buffer p_H 7·6 was prepared, and mixtures of this suspension with phosphate buffer were taken so as to give the amounts of yeast stated in 3 cc. of liquid. In curves A and B the rates of shaking were 102 and 138 oscillations per minute respectively. Temperature 16°.

The maximum rate of uptake which can be measured varies greatly with the rate of shaking, as is to be expected. In order to obtain some idea of this rate of uptake at different rates of shaking, a number of observations were made with larger amounts of yeast, under conditions therefore where diffusion was the limiting factor. These results are shown in Fig. 6. The experimental variations are considerably greater when the process is limited by diffusion than when this is not the case, but the results are quite consistent in spite of the fact that several different respirometers were used. The true respiration rate of the amount of yeast used was about 3000 mm.^{3} per hour at 15°, as shown by determinations on small amounts with rapid shaking. This rate was reached at a rate of shaking of about 180 oscillations per minute.

Rates of shaking below 60 per minute do not cause much mixing of the liquid layer, which tends to oscillate as a whole from side to side of the flask. Definite mixing of the liquid begins at about 60, and thereafter becomes increasingly efficient. This is reflected in the form of the curve. Rates of shaking above 200 are not permissible, as there is then some risk of the liquid splashing on to the "KOH-paper," but there is no risk of this below this rate.

The position of the curve of Fig. 6 is not altogether invariable and independent of the nature and amount of the system absorbing oxygen. Even when the process is limited by diffusion the rate of uptake depends to a certain extent upon the amount and activity of the yeast taken (or, in the case of autoxidation of a substance, the velocity constant of the reaction). In general, systems which would react rapidly with an adequate supply of dissolved oxygen tend to give higher curves than systems giving slower uptakes. That is to say, diffusion effects may influence the results at observed rates much lower in the case of systems giving only small rates of uptake (in the absence of such effects) than in the case of more rapidly reacting systems.

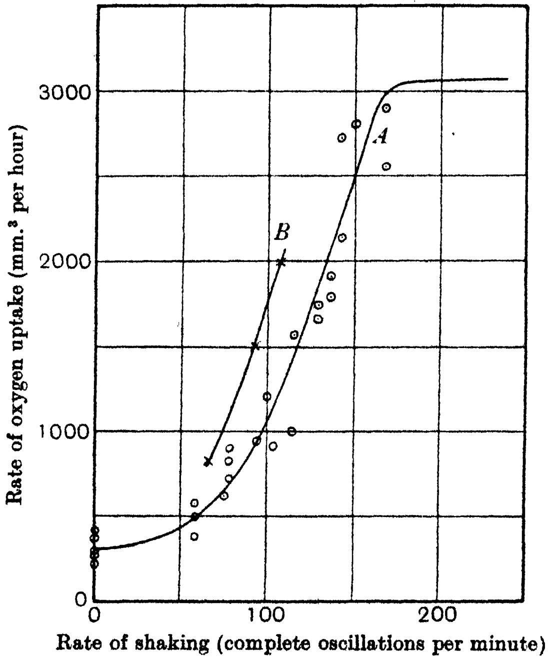

Fig. 6. Effect of rate of shaking on rate of oxygen uptake by large amounts of yeast. Each of the small circles represents an experiment with 600 mg. baker's yeast in 3 cc. phosphate buffer at 15°. The true respiration rate was slightly over 3000 mm.³ per hour. Curve B was obtained with 200 mg. of yeast at 37°.

Thus with smaller amounts of yeast than those used in Fig. 6 the curves followed lie somewhat lower but are of similar form. As the rate of shaking is increased the rate of uptake increases as before until the true respiration rate is reached, after which further increase of shaking has no effect. With decreasing amounts of yeast a series of curves such as E, F, B, C in Fig. 7 are thus obtained, the levels of the horizontal parts of the curves at the higher rates of shaking being proportional to the amount of yeast present. It is of course only on these horizontal parts of the curves that the true respiration rate is measured.

Conversely with more rapidly respiring cells the position of the curve is somewhat higher. In curve D of Fig. 7, for which the same amount of yeast was used as in Fig. 6, the yeast was previously incubated with a small amount of glucose, which increased its respiration about 5 times [cf. Dixon and Elliott, 1929]. The change in position of the curve is however comparatively small.

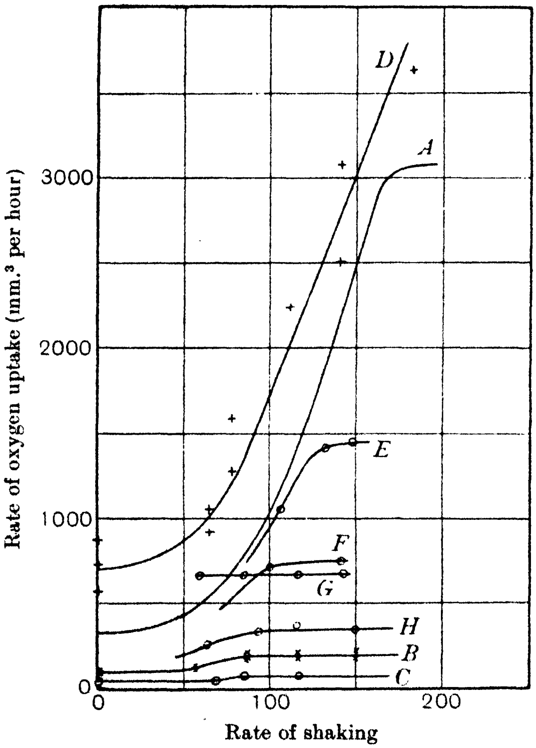

Fig. 7. Curve A is that of Fig. 5 reproduced for comparison. Curves A, B and C were carried out with the same sample of yeast, 600 mg., 60 mg., and 20 mg. being used respectively. Curve D was obtained with 600 mg. of the same yeast which had previously been treated with a small amount of glucose. Curves E and F were obtained with 500 mg. and 250 mg. of a different sample of yeast. Temperature 15° in all these curves. Curve G was obtained with 100 mg. of yeast at 37°, and curve H with 500 mg. of minced liver at 37°.

In the case of yeast the respiration rate is independent of the oxygen pressure within wide limits. In other cases, as for instance the autoxidation of cysteine, the reaction velocity depends upon the oxygen pressure.

A number of experiments were carried out on the oxygen uptake by cysteine in presence of varying amounts of iron as catalyst. The curves obtained were very similar to those given by yeast. It appears that with this system also velocities of the order of 1000 mm.^{3} per hour can be measured at the usual rate of shaking (see Fig. 8).

This figure must not however be taken as representing the highest velocity measurable by the apparatus for all systems. With certain rapid reactions uptake rates of a much higher order can be measured without errors due to diffusion effects. For instance in the case of oxygen uptake by alkaline pyrogallol solutions rates of the order of 10,000 mm.^{3} per hour can be measured at the normal rate of shaking.

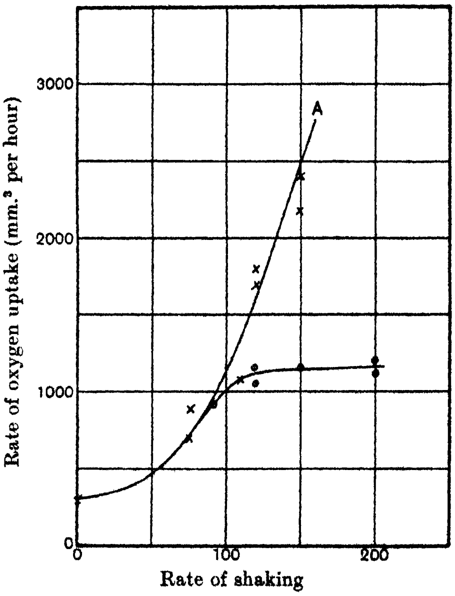

Fig. 8. Effect of rate of shaking on oxygen uptake by cysteine in presence of iron. The curve A is again reproduced from Fig. 6 for comparison. The crosses represent observations with 8 mg. cysteine + 0·04 mg. iron (as $FeSO_4$) in 3 cc. buffer. The circles were obtained with 8 mg. cysteine + 0·016 mg. iron. Temperature 14°.

Fig. 9 shows the rates of uptake observed at different rates of shaking. In these experiments the pyrogallol solution was placed in the flasks, and the NaOH solution was placed in small hanging tubes as shown in Fig. 1. The manometers were then shaken in the thermostat for a few minutes, after which the NaOH was upset into the pyrogallol solution and readings commenced. By this means the rapid uptakes could be conveniently followed.

It will be seen that, although at the slower rates of shaking the reaction was evidently limited by diffusion, the velocity was quite unaffected by increasing the rate of shaking from 120 to 150, indicating that at 120 the true velocity had been reached. This is also indicated by the fact that the velocity of uptake was exactly proportional to the pyrogallol concentration up to even higher velocities, as shown in Fig. 10. We have at present no wholly satisfactory explanation for the difference in behaviour between pyrogallol and the other systems studied.

It is now clear that the limiting rate of uptake which can be measured in the apparatus without being falsified by diffusion effects does not depend only upon the apparatus, but also upon the system under investigation. Our aim in this paper is not to provide a mathematical treatment which will account quantitatively for the effects observed, but rather to call attention to the absolute necessity, when studying any reacting system by means of the apparatus, of making certain that diffusion effects are not affecting the results, if the observations are to have any weight. Two main criteria are available. In the first place, it is obvious that if the reaction velocity is determined for a small amount with rapid shaking this will indicate the true value. If from this the value for a larger amount is then calculated, and the observed value corresponds with the calculated, it is clear that the true rate is still being measured. In other words, if the observed velocity is proportional to the amount of the system taken throughout the experimental range,

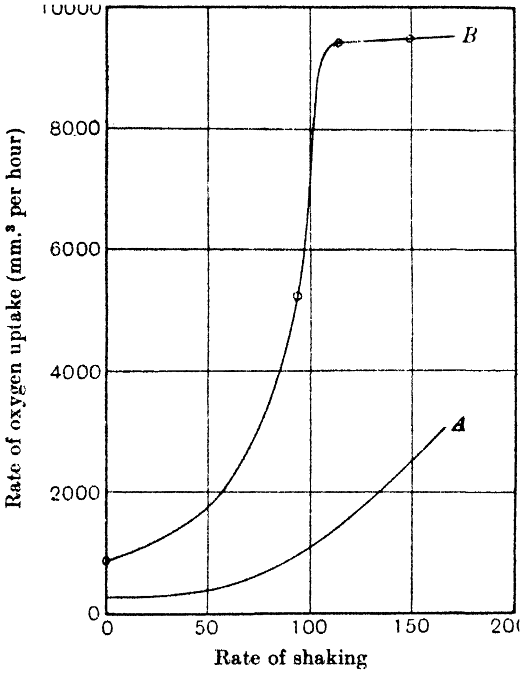

Fig. 9. Effect of rate of shaking on rate of oxygen uptake by alkaline pyrogallol. The curve A is again that of Fig. 6. Curve B was obtained as follows. 2·1 cc. water + 0·5 cc. of 0·5 % pyrogallol solution was placed in the flasks, and 0·4 cc. of 20 % NaOH in the hanging cups. After equilibration of the apparatus the NaOH was upset into the pyrogallol solution. Temperature 14°.

the experiments are valid. The second requirement is that the velocity of uptake must not be affected by the rate of shaking. Unless this is the case it is clear that diffusion effects are entering, and the significance of the results is doubtful. In studying a new system it cannot be predicted from the observed velocity alone whether diffusion effects are influencing the results or not, and one or other of the tests mentioned must be applied.

It can however be stated with some probability that with this type of apparatus at the usual rate of shaking and for reactions of average velocity, diffusion effects do not play a considerable part with rates of uptake less than about 1000 mm.^{3} per hour at 15°. An examination of the literature shows that the rates of uptake measured with this type of apparatus have almost invariably been considerably lower than this—usually about 200–300 mm.^{3} per hour—and in that case it is unlikely that any considerable errors due to this cause have arisen. It is however not altogether uncommon in descriptions of work on oxygen absorption carried out in other types of apparatus to find such statements as "In order to obtain consistent results it was necessary to control carefully the rate of shaking." This of course indicates that such experiments are of doubtful significance.

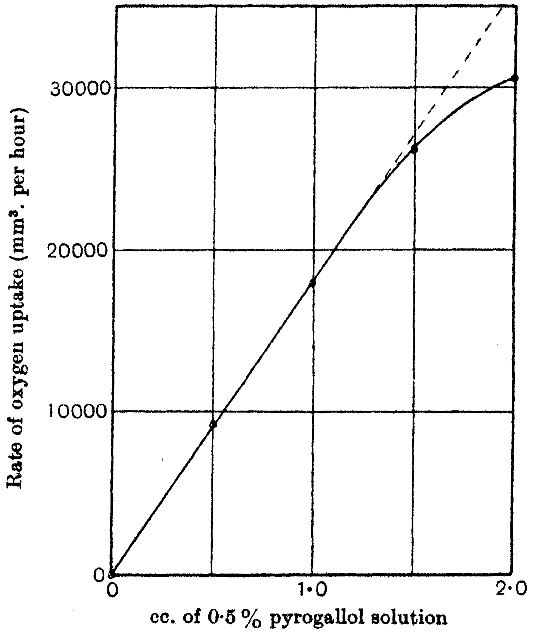

Fig. 10. Effect of amount of pyrogallol used on rate of oxygen uptake. The procedure was the same as in Fig. 9, except that the rate of shaking was maintained constant at 120, and the volume of 0·5 % pyrogallol solution taken was varied. The total volume of liquid was 3 cc. in each case as before.

Finally we may mention that none of our earlier work is affected by these results. We have always applied one or other of the above-mentioned tests to ensure that diffusion errors were eliminated, and when we have worked with systems producing CO_2 we have employed the "KOH-paper" technique. Nevertheless in view of the large amount of work now being carried out with the apparatus it seemed desirable to publish the facts described in this paper.

SUMMARY.

1. The usual method for absorbing the CO_2 evolved in respiration experiments in the Barcroft apparatus is shown to be very inefficient, and an efficient method is described.

2. Experiments on the extent to which diffusion effects may limit the results obtained, when the Barcroft apparatus is used for measuring velocities of oxygen uptake, are described and discussed.

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—— and Tunnicliffe (1923). *Proc. Roy. Soc. Lond.* B **94**, 266.
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XCII. THE OXYGEN CAPACITY OF THE BLOOD IN ONE HUNDRED CASES OF MENTAL DISORDER.

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(*Received April 29th, 1930.*)

It is probable that the most important factor responsible for the abnormal reactions in cases of mental disease can be traced to some variety of defective oxygen metabolism [Pickworth, 1929].

Irregularities of the normal oxygen supply or its utilisation may occur at any point in the respiratory cycle. The present paper deals with deficient oxygen supply due to defective blood-pigment. From pathological considerations it is highly probable that toxic substances absorbed from the intestinal canal (and evidence of blood infection with intestinal organisms is found almost invariably in association with acute mental disorder) might effect a chemical change in the haemoglobin of the blood, rendering it useless for respiratory purposes.

The present investigation was therefore undertaken to ascertain whether inactive forms of haemoglobin occurred in the blood in cases of mental disorder.

The active haemoglobin of a large number of specimens was determined by direct measurement of the oxygen liberated from fully oxygenated blood, and these values were compared with the total haemoglobin content found by other methods of analysis.

Since it was required to measure very small quantities of inactive pigment, it was necessary to devise an accurate and reliable method of analysis specially suited to the work and quantity of material available. A modification of Haldane's blood-gas apparatus [1900, 1920] was made which was adaptable for a small quantity of blood. Control experiments of each factor influencing the analysis were made in order to secure consistency and reliability of the results.

METHODS.

Estimation of the oxygen capacity of blood.

The blood was collected in sterile tubes containing a trace of potassium oxalate and sodium fluoride as an anti-coagulant. Carbon dioxide was removed by exposing in films to a current of moist, carbon dioxide-free air in presence of a phosphate buffer solution of p_H 7·5. A measured quantity

(1 to 2 cc.) of this CO_2-free blood-buffer mixture was introduced into the reaction flask of the apparatus; this was immersed in a water-bath at 37° and gently shaken until the blood had attained equilibrium with the atmosphere in the flask. 1 cc. of ferricyanide with saponin was then admitted from the separate compartment of the apparatus into the reaction flask. The oxygen was completely expelled by shaking the apparatus for 3 minutes, and the volume of gas evolved measured and calculated to 100 cc. of blood.

Description of blood-gas apparatus.

The apparatus consisted of a narrow bore U tube containing clove oil tinted with safranine as a pressure indicator; there was a third limb of wider bore acting as a reservoir for the oil which was also convenient for measuring the gas under definite barometric pressure.

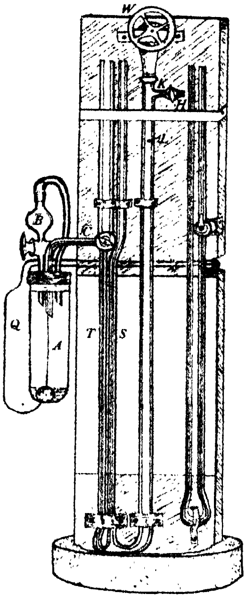

Fig. 1.

The burette *T* (Fig. 1) was fitted with a two-way tap by means of which it could be connected either with the side limb *C* and the reaction flask *A*, or opened to air for adjustment to barometric pressure.

The ferricyanide was contained in a separate compartment *B*, which was connected above with the reaction flask so that it could be admitted without altering the pressure. The barometric pressure and temperature control bulb *Q* was of similar size (about 30 cc.).

The burette tubes *T* and *S* were of identical bore, the former being accurately graduated to a volume of exactly 0·250 cc. and divided into 100 equal parts.

The apparatus was rigidly mounted on a very heavy base and was designed for use completely immersed in a water-bath at 37°. The temperature was controlled by a thermostat and the water was kept thoroughly stirred by a current of air.

The rigid nature of the connections in the apparatus was an important feature and allowed thorough shaking in order to mix the reacting fluids. Complete and rapid expulsion of the liberated oxygen was secured by the introduction of a single large glass marble 1·5 cm. in diameter into the reaction flask.

Accurate and reliable analyses could be made with as little as 0·5 cc. of blood.

A. *Collection and preservation of specimens.*

The blood was collected in sterile tubes containing 0·02 g. of a finely powdered mixture of potassium oxalate and sodium fluoride, 5 : 1.

This anti-coagulant did not cause haemolysis. Estimations were carried out immediately after collection of the specimens when possible. Otherwise they could be stored satisfactorily for a short period in an ice-chamber. It was found that sterile blood, so kept, did not change in oxygen capacity in 4 days, although after 6 days an appreciable diminution was noticeable.

B. *Preliminary treatment of blood samples.*

In order to eliminate errors caused by varying hydrogen ion concentrations of the blood specimens, and also to prevent liberation of carbon dioxide during the test, the p_H of the blood was controlled by means of the addition of strong buffer solution, and the carbon dioxide was removed from the mixture before introducing it into the flask. An equal volume of phosphate solution of p_H 7·5 (containing 8·184 g. $Na_2HPO_4, 12H_2O$ and 0·608 g. NaH_2PO_4 in 50 cc. of distilled water) was added to the blood. A steady stream of air purified from traces of CO_2 by bubbling through dilute sodium hydroxide was passed over the mixture which was gently rotated in a tonometer. This treatment removed about 20 cc. of CO_2 per 100 cc. of blood in 20 minutes. Analyses conducted with the blood so treated were proved to be quite consistent.

C. *Equilibration of samples at 37°.*

For each estimation, 1·6 cc. of the blood-buffer mixture was introduced into the flask *A* by means of a standardised delivery pipette; approximately 1 cc. of recently made ferricyanide (12·5 % dissolved in the same buffer solution, diluted five times in order to bring the salt concentration to a suitable amount), with 0·005 g. of white saponin, was placed in the bulb *B*.

In order for the blood to attain complete equilibrium with the air of the flask, it was necessary to shake the apparatus in the bath for 15 minutes, equilibrium being evidenced by the clove-oil levels remaining quite stationary for 2 minutes.

D. *Process of analysis.*

The levels of the clove oil in the burette tubes were brought to a convenient position near to the zero point by adjusting the pressure in the tube *L*, the temperature gauge was closed, and the ferricyanide solution admitted. The apparatus was gently shaken for 3 minutes. The large glass marble greatly facilitated rapid expulsion of the liberated oxygen in a manner much superior to a number of smaller beads, or broken glass. The levels were adjusted to the gas increase and the temperature of the bath was noted. After obtaining equilibrium the volume of gas was observed and calculated to N.T.P. per 100 cc. of blood. A further shaking of the apparatus was then carried out which should cause no further evolution of gas.

Accuracy of the analysis. We were able to eliminate the following errors by a careful investigation into the conditions under which the test was carried out, and in every case considerable latitude could be allowed in the quantities and times of the tests without introducing these or other errors.

(*a*) Incomplete liberation of oxygen due to faulty haemolysis of the corpuscles (see experimental section E′).

(*b*) Blood laked with dilute ammonia may retain a portion of the liberated oxygen.

(*c*) CO_2 may be liberated owing to the insufficient alkalinity of the solution.

(*d*) Litarczek [1928], and Parsons and Parsons [1927] have described a reducing action which occurs in specimens of pathological blood which results in an absorption of some of the oxygen evolved in the test.

Our experiments under similar conditions demonstrated that some specimens of human blood absorbed a quite appreciable amount of the liberated oxygen. It was found that plasma or serum from normal animals reacted similarly, and even washed rabbit or sheep corpuscles possessed this reducing property to a slight degree. These errors were eliminated by adequately buffering the test mixture, the advantage of which is illustrated by experiments in section B′ and Figs. 2 and 3.

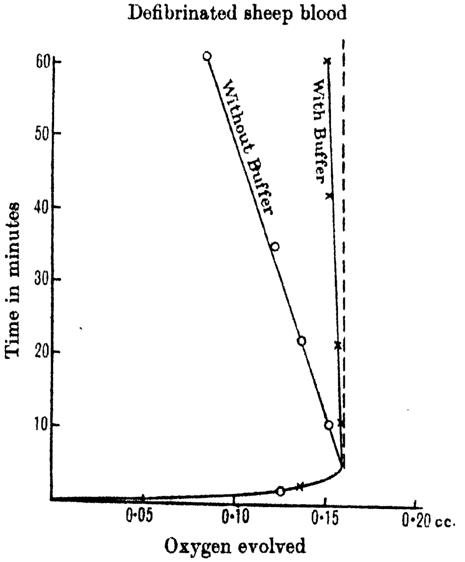

Fig. 2. To show effect of buffer in reducing oxygen absorption in test.

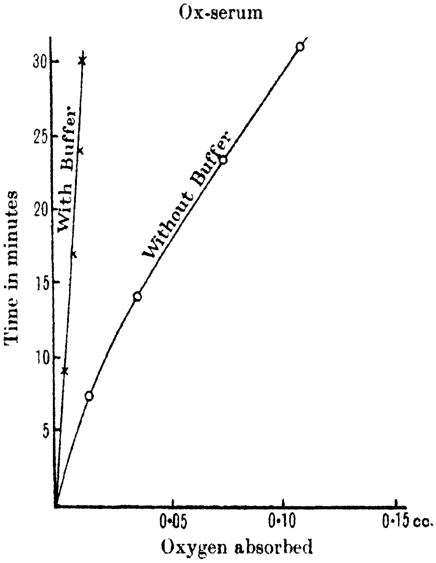

Fig. 3. To show effect of buffer in reducing oxygen absorption in test.

The accuracy of the method was carefully checked by other tests which included Stadie's cyanohaemoglobin colorimetric method, and an estimation of the iron content of the haemoglobin by a micro-titration method using titanium chloride. The quantity of haemoglobin was also estimated by the Fleischl-Miescher haemoglobinometer using a calibrated carbon monoxide wedge.

EXPERIMENTAL.

A′. *Preservation of specimens.*

The following typical experiment demonstrates the change in the oxygen capacity of blood containing oxalate-fluoride anti-coagulant, after storing in the ice-box for various periods.

Specimen No. 59. Sterile rabbit blood + 0·5 % oxalate-fluoride mixture.

Time of storing	Oxygen capacity per 100 cc.
Fresh	15·1 cc.
24 hrs.	15·0 „
48 hrs.	15·0 „
4 days	15·0 „
6 days	12·3 „

No change occurred in 4 days, after which a distinct fall in oxygen capacity took place.

B′. *Effect of buffering the sample.*

1. A large number of sera from various sources, when mixed with an equal quantity of phosphate buffer, were found to have a p_H of 7·5 by the capillator method. The clear fluid obtained by centrifuging a mixture of equal parts of blood and buffer also had a similar p_H value.

2. No alteration in the volume of gas, due to changes in vapour pressure, was caused by the addition of buffered ferricyanide to blood-plasma plus an equal volume of buffer.

Exp. 80. 0·81 cc. human plasma + 0·81 cc. buffer.

Time	Burette reading
Equilibrium	0·185 cc.
3 mins.	0·185 „
Ferricyanide added	
7 „	0·185 „
15 „	0·185 „

3. The addition of acid or alkali to blood did not affect the oxygen capacity, provided the buffer solution as described above was added before saturation with air previous to the test.

Exp. 82. 1 cc. blood + 0·5 cc. 2 % Na_2CO_3; oxygen evolved after buffering etc. = 0·120 cc.

1 cc. blood + 0·5 cc. 0·3 % lactic acid; oxygen evolved after buffering etc. = 0·120 cc.

1 cc. blood + 0·5 cc. isotonic saline; oxygen evolved = 0·121 cc.

4. The addition of a small amount of lactic acid to blood has a very marked effect at low oxygen pressures; when this lactic acid-blood is treated with buffer prior to saturation with the low pressure of oxygen, the calculated theoretical result is obtained.

Exp. 83. 1 cc. corpuscles + 1 cc. isotonic saline + 0·5 cc. 0·1 % lactic acid exposed to 2·6 % oxygen in nitrogen.

Oxygen evolved = 0·194 cc. = 67·5 % saturated.

(Calculated saturation = 84 %.)

1 cc. corpuscles + 0·5 cc. lactic acid + 1 cc. buffer solution, then exposed to 2·6 % oxygen in nitrogen.

Oxygen evolved = 0·225 cc. = 84 % saturated.

(Calculated saturation = 84 %.)

5. The effect of the buffer in preventing the re-absorption of oxygen by the blood-ferricyanide mixture is shown by the following experiment.

Exp. 85. 1 cc. sheep plasma + 1 cc. 2 % Na_2CO_3, after equilibrium established with its atmosphere in the reaction flask, 1 cc. aqueous ferricyanide added. Readings taken at intervals.

Time	Burette reading	Time	Burette reading
0 mins.	0·229 cc.	12 mins.	0·193 cc.
2 „	0·220 „	18 „	0·183 „
5 „	0·212 „	25 „	0·170 „
8 „	0·200 „	30 „	0·162 „

Absorption in 30 mins. = 0·067 cc.

Exp. 86. 1 cc. sheep plasma + 1 cc. buffer p_{H} 7·5; after equilibrium established, 1 cc. buffered ferricyanide added; readings at intervals.

Time	Burette reading	Time	Burette reading
0 mins.	0·190 cc.	15 mins.	0·188 cc.
5 „	0·189 „	30 „	0·188 „
10 „	0·189 „	40 „	0·187 „

Oxygen absorbed = 0·003 cc.

Similar tests comparing alkaline with buffered solutions are shown in Figs. 2 and 3.

It will be noted that the serum is responsible for the greatest re-absorption of oxygen. A further similar test with washed sheep corpuscles gave a much smaller absorption deviation.

C′. *CO_2 in the sample.*

When considerable amounts of carbon dioxide are present in the blood sample, a portion of it passes into the air above the mixture in the reaction flask. This quantity may vary slightly on adding the reagent, and samples must therefore be previously treated to remove excess CO_2.

Exp. 87. (*a*) 1 cc. sheep blood-plasma previously exposed to CO_2-free air current for 20 minutes (CO_2 content = 20 cc./100 cc.); 1 cc. buffer added; ferricyanide admitted when in equilibrium.

	Time	Burette reading
	0 mins.	0·147 cc.
	3 „	0·147 „
	Equilibrium established	
Ferricyanide added	5 mins.	0·147 cc.
	10 „	0·146 „
	25 „	0·146 „

No change on adding reagent.

(*b*) 1 cc. sheep plasma (untreated), CO_2 content = 40 cc./100 cc., 1 cc. buffer added; ferricyanide admitted when in equilibrium.

Time	Burette reading
0 mins.	0·135 cc.
3 ,,	0·135 ,,
Ferricyanide added	
5 ,,	0·135 ,,
8 ,,	0·120 ,,
10 ,,	0·116 ,,
12 ,,	0·116 ,,

When blood-plasma containing a considerable quantity of carbon dioxide is used, there is a distinct change in the volume of the gas on addition of the reagent, due to absorption of CO_2.

Exp. 88. The quantity of CO_2 in the atmosphere of the reaction flask is shown in the table below. Blood + equal volume of buffer p_H 7·5 exposed for varying periods to a current of moist, CO_2-free air; 2 cc. of the resulting mixture introduced into the apparatus. After the volume had become constant, the CO_2 in the flask was absorbed by admitting 0·2 cc. of 5 % sodium hydroxide, the contraction in volume being the amount of CO_2 present.

Time of exposure to CO_2-free air	CO_2 in gas of reaction flask	Equivalent mm. of CO_2
0 mins.	0·57 %	3·9 mm.
5 ,,	0·31 %	2·2 ,,
10 ,,	0·21 %	1·5 ,,
20 ,,	0·14 %	1·0 ,,
30 ,,	0·13 %	0·91 ,,
60 ,,	0·12 %	0·85 ,,

Laboratory air = 0·4 mm. CO_2.

Exp. 89. Elimination of excess CO_2. The blood at p_H 7·5 was exposed in a tonometer through which a current of CO_2-free air was passed for varying periods.

Time of exposure	CO_2 per 100 cc. blood
0 mins.	42·5 cc.
1 ,,	37·5 ,,
5 ,,	28·0 ,,
15 ,,	22·5 ,,
20 ,,	21·2 ,,
30 ,,	20·0 ,,
3 hrs.	17·5 ,,

An exposure of 20 minutes was sufficient to remove the excess CO_2.

D′. *Equilibration of gas pressures.*

It was found that whole blood required about a quarter of an hour to attain equilibrium with the air of the reaction flask, gentle shaking of the apparatus being continued during the whole time. This period was much increased for plasma, whilst only a few minutes were required for a suspension of washed blood-corpuscles in saline.

E′. *The ferricyanide-saponin reagent.*

This consisted of 12·5 g. ferricyanide + 0·2 g. white saponin in 100 cc. phosphate buffer diluted five times with distilled water.

1. *Amount of ferricyanide.* A large excess of the ferricyanide mixture is necessary in order to decompose the oxyhaemoglobin completely. (Conant and Fieser [1924] have calculated that at least 13·5 equivalents per g.-molecule of haemoglobin are required when in neutral solution.)

The following test shows that the amount used (0·125 g. for each estimation) was amply sufficient, and that this amount could be varied considerably either way without error.

Exp. 90. (1) 2 cc. buffered ox blood + 1·5 cc. ferricyanide reagent: oxygen evolved = 0·216 cc.
(2) 2 cc. buffered ox blood + 1·0 cc. ferricyanide reagent: oxygen evolved = 0·216 cc.
(3) 2 cc. buffered ox blood + 0·5 cc. ferricyanide reagent: oxygen evolved = 0·216 cc.

2. *Amount of saponin.* The addition of an amount of saponin equivalent to that in the reagent (*i.e.* 0·002 g. per cc.) to a sample of blood caused complete haemolysis in 2 minutes. Microscopic examination of a drop of the fluid failed to show the presence of corpuscles. The addition of strong salt solution to this clear fluid did not cause the re-appearance of corpuscles (microscopically or by turbidity) as occurs with blood laked with a small quantity of distilled water.

The following test shows that the accuracy of the analysis was not affected if the amount of saponin added to the blood varied considerably from the quantity actually employed.

Exp. 92. (1) 2 cc. buffered ox blood + 1·0 cc. ferricyanide containing 0·4 % saponin: oxygen evolved = 0·203 cc.
(2) 2 cc. buffered ox blood + 1·0 cc. ferricyanide containing 0·2 % saponin: oxygen evolved = 0·203 cc.
(3) 2 cc. buffered ox blood + 1·0 cc. ferricyanide containing 0·15 % saponin: oxygen evolved = 0·203 cc.

The rate of absorption of oxygen by haemoglobin was markedly slower in the presence of saponin.

Exp. 93. (1) Human blood exposed to 2·1 % oxygen. Then exposed to air for 15 mins.
Oxygen content = 17·8 cc. per 100 cc. = 100 % saturated.
(2) Human blood exposed to 2·1 % oxygen; saponin added. Exposed to air for 15 mins. exactly as in test (1).
Oxygen content = 16·4 cc. per 100 cc. = 92 % saturated.

Other methods of estimation of haemoglobin content.

1. *Cyanohaemoglobin colorimetric determination.* A slight modification of the process described by Stadie [1920] and Van Slyke and Stadie [1921] has been utilised. The pigments are converted to methaemoglobin by adding a trace of ferricyanide to the diluted blood and then, by adding a little dilute

potassium cyanide solution, cyanohaemoglobin is produced which has a brownish-red colour suitable for colorimetric estimation. A control is used, similarly prepared from pure blood. Blood containing small amounts of methaemoglobin shows a higher value for the colorimetric, as compared with the gasometric, method.

0·2 cc. blood is diluted to 5 cc. with distilled water; 1 drop of 10 % ferricyanide containing 2 % lactic acid is added. After standing for 15 mins., 1 cc. 0·1 % KCN is added and the solution diluted to 20 cc. The resulting colour is matched with the standard similarly prepared.

The lactic acid greatly facilitates the rate of conversion to methaemoglobin, and the colour is stable and unaffected by the amount of acid present.

2. *Fleischl-Miescher haemoglobinometer method.* A thin film of blood was exposed to coal gas in a tonometer until saturated with carbon monoxide. It was diluted 1 : 200 in a haemocytometer pipette with water saturated with carbon monoxide and the solution placed in one-half of the comparing chamber, the other half being filled with distilled water. The haemoglobin values were read directly from a carbon monoxide wedge.

3. *Micro-titration of the iron content.* This method of analysis was used to check the other determinations and is similar to that used by Peters [1912].

The blood was weighed into a small nickel crucible (about 0·3 g.) and ashed by direct heat. The residue was dissolved in 2 drops of distilled water and 1 drop of strong iron-free hydrochloric acid. A drop of dilute hydrogen peroxide was added, the solution diluted to about 0·5 cc. and evaporated almost to dryness in order to ensure decomposition of all traces of peroxide. The solution was transferred to a titration tube having a constricted neck and bulb of capacity 3 cc. with a little acidulated water, and the volume of solution and washings made up to 2 cc. The titrations were made from a micro-burette, capacity 1 cc., with approx. $N/200$ titanium chloride. The reagent was made by adding 15 cc. of 15 % commercial titanium chloride to 15 cc. of hydrochloric acid, boiling for 3 mins. and diluting to 2500 cc. with well-boiled water. This volume completely filled the containing vessel.

It was stored in contact with an atmosphere of CO_2, and standardised immediately before making analyses.

In order to prevent re-oxidation by the oxygen in the titration tube, the air was completely displaced before the analysis by means of a rapid stream of CO_2 and the surface of the liquid was covered with a thin layer of liquid paraffin.

One drop of 10 % KCNS was used as indicator and the titanium chloride was added from the micro-burette until the red colour just disappeared. When the ashing process was correctly performed and the precautions indicated above were observed, no return of the red tint occurred for some time and accurate analyses were obtained.

RESULTS.

Blood specimens from a number of normal healthy persons and 130 specimens from 100 patients have been analysed. Also 40 specimens from healthy animals, including the sheep, ox, pig and rabbit, have been determined, and a number of analyses have been made with the blood of cats and rabbits whilst under treatment with drugs.

All the analyses were performed in duplicate, the results agreeing to within 2 % for the colorimetric tests and 1 % with the other determinations. The following is a summary of the results obtained. Values have been expressed on the basis of 18·5 cc. oxygen = 100 % haemoglobin (Haldane's standard for normal male).

A. *Normal human blood.*

The following tables show the results obtained in analyses of the blood of a number of healthy persons. A repeat of No. 1 is included to show the consistency of results obtainable after a few hours' interval.

Table I. *Oxygen capacity (normal human specimens).*

Specimen	Oxygen evolved per 100 cc.	Corresponding % Hb. content
1	19·0 cc.	102·6
2	18·6 „	100·7
3	17·6 „	95
4	18·4 „	99·5
1 (repeat)	19·05 cc.	102·9

Table I *a*. *Haemoglobin content (comparison of methods).*

Specimen	% Hb. by gasometric method	% Hb. by Stadie method	% Hb. by CO-Hb. method
1	102·6	103	102
2	100·7	101	100
3	95·1	Standard	94
4	99·5	99·2	98

Table II gives the results of analyses of a number of specimens from healthy animals.

Table II. *Haemoglobin content of animals' blood.*

Animal	% Hb. by gasometric method	% Hb. by $TiCl_3$ titration	% Hb. by CO-Hb. method	% Hb. by Stadie method
Sheep	108	106·5	106	108
Ox	86·5	86·8	87	Standard
Pig	71·4	72·2	70	71·2
Ox (corpuscles)	99·4	—	99	99·3
Rabbit	59·5	—	59	59·2
Cat	57·8	—	58	57·2
Sheep	110·8	109	110	110·7
Pig	84·3	86	85	84

The values calculated from the gasometric method agreed very closely in all cases with those by the cyanohaemoglobin colorimetric method. The values by the Fleischl-Miescher method varied slightly owing to a greater difficulty in matching the colour. (The tints of the blood solution, when fully saturated with carbon monoxide, compared satisfactorily with the standard wedge with the exception of rabbits' blood which gave a yellower tint.) Determinations of the exact colours were also made by the Rosenheim-Schuster colorimeter, and some of the results are given which may possibly be useful for reference.

Colour values of haemoglobin solutions.

Normal human blood diluted 1 : 100

Specimen	Tintometer units
Oxyhaemoglobin	Red 15·0; yellow 1·5
Carboxyhaemoglobin	Red 20·2; yellow 0·2
Cyanohaemoglobin	Red 12·0; yellow 4·2

B. *Patients' blood.*

The absolute haemoglobin content as found by the gasometric method is given in Table III.

Table III. *Haemoglobin content of patients' specimens.*

	Males		Females	
Hb. content	No. of specimens	%	No. of specimens	%
Over 100 %	6	16	9	10
90–100 %	20	53	30	32
80– 90 %	9	23	39	42
70– 80 %	3	8	11	12
Below 70 %	0	0	3	3

In only comparatively few cases was there any serious deficiency in the quantity of available haemoglobin. The average haemoglobin content for the whole series was 93 % for males and 89 % for females.

Table IV gives the results in 20 unselected cases, 10 male and 10 female.

Table IV.

Sex	Oxygen from 100 cc. blood cc.	% Hb. by oxygen evolved	% Hb. by cyano-Hb. method	% Hb. by CO-Hb. method	Notes
M.	16·2	87·6	88	86	—
,,	16·1	87·0	88	88	—
,,	17·6	95·3	95·6	95	—
,,	16·5	89·2	96·5	96	7·3 % inactive Hb.
,,	18·0	97	97	98	—
,,	19·8	107	107	108	—
,,	17·5	95	94	93	—
,,	17·0	92	98	96	6 % inactive Hb.
,,	17·1	92·6	93	93	—
,,	16·6	90·0	91	90	—
F.	17·0	92	92	92	—
,,	16·0	86	86	86	—
,,	15·9	86	87	87	—
,,	20·1	108·5	110	110	1·5 % inactive Hb.
,,	15·5	84	84·8	85	—
,,	19·6	106	106	106	—
,,	16·4	88·5	92	91	3·5 % inactive Hb.
,,	17·5	95	93·5	95	—
,,	15·9	86	86	86	—
,,	16·5	89	89	88	—

Three of the above specimens gave low results by the oxygen method as compared with the colorimetric and titrimetric methods; this indicates the presence of inactive haemoglobin.

Among the total 100 cases, 28 showed deficient oxygen capacity compared with the theoretical. In all these the difference exceeded 2·5 %, the greatest deficiency being 12·5 %, which was observed in two specimens; neither of these patients had received treatment with sulphonal or allied drugs.

In accordance with previous supposition of the effect of intestinal disease, the 100 cases contained a large number whose blood showed a positive agglutination by the Widal test. (See Table VI.)

A number of cases showing deficient active haemoglobin were examined at intervals of several weeks. In most cases the deficiency persisted for a long period. The following table is included to illustrate this important finding.

Table V. *Haemoglobin in blood of special cases.*

Name	Date	Sex	% Hb. by gasometric method	% Hb. by colorimetric method	% difference
H.F.	14. iii. 29	F.	95	100	5
„	3. v. 29	„	89	97	8
„	30. v. 29		99·3	109	9·7
„	14. vi. 29		101	113	12
A.S.	24. v. 29		72·6	83	10·4
„	14. vi. 29		74	80	6
A.J.	14. vi. 29		80	86·5	6·5
„	16. v. 29		71·5	82	10·5
B.W.	19. vii. 29		90	99	9
„	25. vii. 29		81	87·3	6·3
„	30. viii. 29		85·1	92	6·9
„	5. ix. 29		91	102·8	11·8
D.G.	30. viii. 29		82·4	92	9·6
„	5. ix. 29		84	89	5
„	18. ix. 29		101·3	107·1	5·8
L.S.	1. viii. 29		90	100·8	10·8
„	5. ix. 29		79	88	9
D.R.	5. vii. 29	M	92	98	6
„	2. viii. 29		92·5	98	5·5
R.P.	27. vi. 29		89·2	96·5	7·3
„	2. viii. 29		85	91	6·0
J.G.	5. ix. 29		95	107	12·0
H.H.	26. ix. 29		103	110	7·0

Table VI.

Widal agglutination reaction	No. of cases	Cases with over 2 % of inactive pigment	Average % of inactive pigment
+	52	27	4·2
–	38	1	0·38

A simple test for methaemoglobin.

The following test demonstrates the presence of methaemoglobin when 5 % or more is present in blood; with the simple type of spectroscope about 20 % of methaemoglobin must be present for reliable detection.

0·20 cc. of blood is saturated with carbon monoxide (coal-gas) in a tonometer and then diluted with 40 cc. water saturated with carbon monoxide. 10 cc. are transferred to each of two similar tubes and 1 drop of 2 % ammonia is added to each. The air is displaced by coal-gas from the tubes, which are corked and shielded from bright light. To one tube 0·5 cc. of 1 % KCN is added, and to the other 0·5 cc. of freshly made solution of 1 % sodium hydrosulphite, avoiding undue exposure to air. The stoppers are replaced and the tubes gently inverted. The methaemoglobin of the one tube is converted to brownish-red cyanohaemoglobin, and the methaemoglobin in the other is first reduced to haemoglobin followed by formation of carbon monoxide-haemoglobin. The colour difference is recognised by naked-eye comparison. The colours produced were quite stable when air was excluded.

Discussion.

The presence of inactive haemoglobin has been found to occur in one quarter of the cases examined, the amount in two cases being as much as 12 %. It is submitted that the amount of inactive haemoglobin under certain circumstances is very much higher than this, and further work is being continued with acute cases.

The presence of inactive haemoglobin has been correlated with diseased conditions of the intestinal tract. Such diseased conditions are very frequent in cases of mental disorder, and evidence of blood infection apart from clinical disease can be obtained from the agglutination reaction of the blood in about one quarter of the cases admitted to the mental hospitals investigated (Birmingham) [Pickworth, 1927, 1928].

Table VI shows the relation of inactive haemoglobin to a positive agglutination reaction. The close correlation is obviously more than coincidence and tends to confirm the view that an infected intestinal tract allows the absorption over a large area of bacteria and toxic substances which deprive the blood of its primary function, that of oxygenation. Other visceral conditions of focal sepsis of the upper respiratory tract, the fatty liver and kidneys and the atheroma of the blood vessels, so often found in cases of mental disorder, support this view.

Buscaino [1923, 1926] has also described lesions of the small intestine and holds that a toxic substance is absorbed, especially in acute confusional insanity and in dementia praecox. He suggests that in these cases the liver does not function normally and that amino-acids become decarboxylated, giving rise to poisonous amino-compounds. Stewart [1929] suggested that this toxic compound is tyramine and demonstrated that tyrosine was destroyed with production of the amine by the intestinal bacteria from cases of acute insanity.

Boyd [1923] found histamine in the intestines of children dying from intestinal toxaemia. Looney [1924] has described toxic amines in the blood of mental patients in the depressed phase.

A number of animal experiments have been conducted with a view to reproducing some of these conditions.

1. *Effect of chemical reagents.* (*a*) A rabbit, 2000 g., was injected intravenously with the following quantities of sodium nitrite at intervals of 30 minutes: 0·25, 0·5, 1·0, 1·5, 2·0 cc. of 2 % solution, 2·0 cc. of 5 % solution. Blood samples were taken after 12 hours. The haemoglobin value was 70 %, but no inactive haemoglobin was demonstrated. (Sodium nitrite readily produces methaemoglobin *in vitro*.) This experiment shows how quickly methaemoglobin disappears from the blood. Haldane found that methaemoglobin disappeared in about 4 hours.

(*b*) A rabbit, 1500 g., was injected intravenously with 5 cc. of 5 % sodium nitrite solution. A blood sample taken 40 minutes later contained 64·2 % of blood-pigment by the Stadie method. Only 21·6 % was oxyhaemoglobin, the capacity being 4·0 cc. O_2 per 100 cc. Thus 42·6 % of methaemoglobin was present. The blood was dark and when saturated with carbon monoxide yielded a brown-coloured solution. The animal died shortly after.

(*c*) A cat, 900 g., was given 5 grains phenacetin by mouth on five successive days. The total haemoglobin content of samples taken after the last dose was 45 %, and the active pigment amounted to only 34 % (8·4 cc. oxygen per 100 cc. blood). Thus 11 % of inactive material was present. The blood was dark and the animal anaemic. The presence of the inactive pigment was also demonstrated by the special test described. (Young [1926] asserts that aniline and certain derivatives do not produce methaemoglobin when administered to animals and that the dark colour of the blood in such cases is due to *p*-aminophenol.)

2. *Effect of bacterial injections.* (*a*) Experiments were made in which emulsions of *Streptococcus mitis* and *S. faecalis* were injected intravenously into rabbits. In neither case was there any indication of inactive pigment in the blood.

(*b*) An emulsion of 0·2 cc. of *B. nitrosobacter* was injected intravenously into a rabbit. A specimen of blood taken 2 days later had an oxygen capacity of 11·7 cc. O_2 per 100 cc., *i.e.* 63 % of the normal Hb. content. The total haemoglobin by the Stadie method was 70 %, *i.e.* 7 % of inactive haemoglobin was present.

(*c*) The following injections of T.A.B. vaccine (2000×10^6 organisms per cc.) were made intravenously into a rabbit of 2000 g. weight. 3 cc. on 3. xii. 29, 6 cc. on 9. xii. 29, 12 cc. on 17. ix. 29, 20 cc. on 23. ix. 29. After each injection samples of blood were examined, but in no specimen was inactive pigment demonstrated. In spite of the enormous doses the animal remained apparently well.

(*d*) A rabbit, 2000 g., was injected with 5 cc. of emulsion of *B. enteritidis* Gaertner (20×10^9 organisms per cc.). A blood sample taken 24 hours after the injection contained 15 % of inactive haemoglobin, the active oxyhaemoglobin being 54 %. The animal died 40 hours after the injection.

The nature of the inactive pigment.

The chemical properties of the inert pigment are similar to those of methaemoglobin, and differences, if they exist, can only be ascertained by complicated quantitative spectroscopic analysis. It does not yield oxygen to ferricyanide or a vacuum, is unaffected by carbon monoxide, and has a brownish colour; it is reduced by sodium hydrosulphite.

The occurrence of similar pigment in pathological blood has been described by a number of workers. Harrop [1919], in describing two cases of pneumonia, concluded that methaemoglobin was responsible for the deficiency in oxygen capacity. Haldane [1929] attributed the fatal character of the influenza epidemic of 1918 to the production of methaemoglobin by the infecting organisms.

Parahaematin or cathaemoglobin has been described by Arnold [1900], v. Klaverin [1901] and by Keilin [1926]. This substance does not yield oxygen to a vacuum or to ferricyanide, but possesses a spectrum very similar to oxyhaemoglobin.

Loeb, Bock and Fitz [1921] describe two cases of nitrobenzene poisoning in which the oxygen capacity by the colour should have been 20 cc. O_2 per 100 cc. blood. Actually the values 6·2 and 8·9 cc. were obtained, but no methaemoglobin could be detected spectroscopically. Peabody [1913] also found a fall in the oxygen capacity of guinea-pigs after injection with massive doses of pneumococcus. No spectroscopic evidence of methaemoglobin could be found.

Ray [1928], Ray and Stimson [1927], Stimson [1927], and Stimson and Houbitz [1928] have observed an inactive substance in the blood of rabbits and dogs after removal of the spleen or 80 % of the liver and also following administration of nitrobenzene into the stomach. The maximum amount found was 15 %. It disappeared from the blood after 7 hours in the nitrobenzene experiments and not until 5 days following splenectomy.

Carbon monoxide-haemoglobin, sulph-haemoglobin, and nitric oxide-haemoglobin, which are useless for respiratory purposes, have been sought for but were not found in any of the specimens.

SUMMARY.

The oxygen capacity of blood has been compared with the total haemoglobin content in order to find out whether derivatives of haemoglobin, useless for respiratory purposes, occur in the blood of mental patients.

A special form of apparatus of the Haldane type has been devised, yielding results of greater accuracy and reliability than heretofore.

The haemoglobin was determined by the Stadie cyanohaemoglobin method, the Fleischl-Miescher haemoglobinometer and a titration of the actual iron content by the titanium chloride method.

130 specimens from 100 cases have been examined, and in 28 cases deficient oxygen capacity has been recognised. The difference between the active and total haemoglobin varied from 2·5 to 12·5 %.

The presence of inactive pigment can, in many instances, be correlated with positive Widal agglutination reactions to organisms of the enteric group, and these infections may prove of importance in the aetiology of this pigment.

The above work has been carried out in the laboratories of the Joint Board of Research for Mental Diseases, City and University of Birmingham.

Our thanks are due to Professor J. S. Haldane and Mr Ivon Graham for their help in the perfecting of the apparatus employed, and also to Dr T. C. Graves, Superintendent of the Birmingham City Mental Hospitals and the Medical Officers of the Institutions, for facilities in obtaining the specimens.

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BIOCHEMICAL JOURNAL, 1930

(Volume 24)

ERRATA

P. 365 *footnote*

"Curve B" *should read* "Curve C".

P. 372 *Table* 1

In columns "Precipitate-N" *and* "Filtrate-N" *the headings* "%" *and* "mg." *should be reversed.*

P. 376 *line* 4

"Arginine 35·4 mg." *should read* "Arginine 351·4 mg."

P. 607 *line* 2 *from top*

For "2 minutes" *read* "20 minutes."

XCIII. THE NATURE OF THE BLOOD-SUGAR.

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(*Received April 8th, 1930.*)

Isolation of the blood-sugar has been seldom attempted. Hédon [1898] worked with the blood of depancreatised dogs. The proteins were separated by dialysis for several days at low temperature, and the solution containing the sugar was evaporated to a syrup in open vessels at 60–70°. Partial purification was effected by precipitation with neutral lead acetate; the filtrate after removal of lead was evaporated. The solid precipitated from the syrup by addition of ether gave glucosazone on heating with phenylhydrazine. The reducing power determined by titration with Fehling's solution showed a considerably greater glucose content than that deduced from the optical activity. Similar results were obtained with the blood-sugar of the horse; the glucose content estimated by the polarimeter was rather more than half that found by titration. Hédon found substantial agreement between the two methods in the case of sugar prepared from urine in diabetes, and concluded that the urinary sugar differed from that of the blood, undergoing some modification at the moment of its secretion. Hanriot [1898] used horse blood, which was deproteinised by boiling with sodium sulphate; the filtrate was concentrated and the syrup purified by repeatedly dissolving in methyl alcohol and precipitating with ether. A dry powder was obtained, and when the sugar was estimated by Fehling's solution or polarimetrically there was good agreement. The sugar could be converted into glucosazone or parachloralose. Neither Hédon nor Hanriot records analyses or melting-points of the substances isolated by them, nor was the sugar pure enough to show mutarotation. The sugar content given by the polarimeter was deduced from the equilibrium value of α- and β-glucose. This value was also used in the calculation of the sugar content of blood-filtrates by Winter and Smith [1923] and compared with that found by estimation of the copper-reducing power. It was found that blood-filtrates from normal men and animals showed a marked discrepancy between the figures obtained by the two methods, but that this difference disappeared in the course of some days. On the other hand, in diabetes the values agreed from the outset, and it was suggested that the sugar was of a different type in the two cases. Subsequently, Lundsgaard and Holbøll [1925] came to a similar conclusion. Denis and Hume [1924] repeated the experiments of Winter and Smith [1923] and concluded that the changes in optical activity on keeping the sugar solutions were due to contamination with micro-organisms; the changes recorded by them were, however, in the opposite direction. In all the above experiments observations were made on sugar solutions contaminated with varying amounts of other substances. The object of the present work was to separate the sugar from normal blood in such a way as to enable it to be identified with certainty.

EXPERIMENTAL.

The blood of the rabbit has been used throughout. The animal having been killed by a blow on the head, the throat is cut and the blood received into a shallow basin. The blood is defibrinated by whipping and the volume measured. The proteins are precipitated with the Folin-Wu reagents, seven volumes of water being used for dilution. After vigorous shaking the mixture is allowed to stand for an hour and then filtered through papers which have been very thoroughly washed with water. When filtration is complete the precipitate is washed with a little cold water. The total volume of filtrate measures about 800 cc. for 100 cc. blood. Concentration, which is carried out *in vacuo* at 35–40° until the volume is rather more than half the original volume of blood, should be completed in 2–3 hours. The concentrated solution is transferred to a flask, and the distilling flask washed out with saturated lead acetate, 0·5 cc. per cc. blood. The lead acetate is added to the sugar solution. After shaking, the mixture is allowed to stand for an hour. The bulky precipitate is centrifuged, and washed with small amounts of water. The clear solution is cooled in ice, and made alkaline with strong ammonia. The precipitate is allowed to settle for a few hours, being kept cold, and then separated by the centrifuge. A fifth of the previous quantity of lead acetate is added to the fluid, and a further small amount of ammonia. The second precipitate is centrifuged in the same cups as contain the first. The precipitate is stirred up with 80 % alcohol until all lumps have disappeared and then spun down. The washing is repeated. The cups and precipitate are dried in a vacuum over sulphuric acid. The lead compound may be kept for some time in the dry state if convenient. The dry precipitate is weighed and replaced in a centrifuge cup. For each gram 2 cc. 2*N* sulphuric acid are added, and the precipitate is disintegrated with a glass rod; almost continuous stirring for 10 minutes is advisable for complete decomposition. If not acid to litmus further acid is cautiously added, with stirring, until a test sample is distinctly acid. The fluid is separated in the centrifuge and transferred to a measuring cylinder. The precipitate is stirred with a small amount of water, separated, and the washing added to the first solution. The volume is noted. The solution is transferred to a small flask, and 4 volumes of strong alcohol are added gradually, shaking between each addition. The lead precipitate is washed twice with 5 cc. of 80 % alcohol, and the washings are added to the flask. It is advisable to allow a day in the cold chamber for complete precipitation; haste at this stage is liable to cause difficulty later. The solution of the sugar in alcohol is centrifuged, and the fluid, which should be quite clear, is separated. It is better not to wash the precipitate as there is a tendency to form a colloidal suspension. The solution is cooled in ice, and a slight excess of 10 % baryta in methyl alcohol is added. After standing for an hour in ice, the barium compound is separated in the centrifuge and washed twice with cold 80 % alcohol. The precipitate is suspended in 80–100 cc. of 80 % alcohol, warmed to 40°, and decomposed with a rapid stream of CO_2 for 2 hours. After 24 hours in the cold room the carbonate is filtered off, and the solution completely evaporated under reduced pressure. To the distilling flask 15 cc. of 80 % alcohol are added, a trace of insoluble matter is filtered off, and the solution allowed to evaporate slowly in a small basin over sulphuric acid. There is left a colourless syrup which begins to crystallise on stirring. Very soon there is a hard crystalline mass which is removed, rapidly washed with alcohol, and dried. The yield from 100 cc. blood averages 36 mg. To obtain pure α-glucose the crude product is twice recrystallised from dry methyl alcohol, best by allowing the solvent to evaporate very slowly at room temperature; the sugar crystallises in fine needles which are washed with alcohol and dried in a vacuum over sulphuric acid.

Melting point. 145–146° (uncorr.).

Analysis. (Micro-analyses by Dr Ing. A. Schoeller, Berlin.)

C 39·87, 39·73 %; H 6·71, 6·64 %.
$C_6H_{12}O_6$ requires C 40·00 %; H 6·66 %.

Optical activity. All readings were taken in a 1 dm. tube of 4 mm. bore. Only 1·5 cc. of fluid are required, and it is possible with this tube to obtain as great a degree of accuracy as when a tube of standard bore is used. The polarimeter was a three field instrument; a half shadow angle of 1° was used. All readings agreed within 0·02°. The substance shows mutarotation in water and dry methyl alcohol; the rate of change in the latter solvent is slow. 17·4 mg. were dissolved in 1·6010 g. water. $c = 1·08$.

Time (hrs.)	0·08	1	1·5	3·1	9·0	18
α	+1·36	1·00	0·90	0·80	0·66	0·66°
$[\alpha]^{14°}_{5461}$	+125	92	83	74	61	61°

8·4 mg. were dissolved in 1·6155 g. methyl alcohol (*d.* 0·799). $c = 0·41$.

Time (hrs.)	0·08	7	22	44
α	+0·56	0·52	0·50	0·46°
$[\alpha]^{14°}_{5461}$	+136	126	121	112°

Molecular weight. Determinations were carried out by Barger's method. Methyl alcohol containing 25 % of water was used as solvent, and urea as standard. 10·5 mg. sugar were dissolved in 0·522 g. methyl alcohol of *d.* 0·878, giving a concentration of 17·6 g. per litre. This solution was sealed in capillary tubes with the urea solutions. Tube 1 contained 0·09 *M*, tube 2, 0·1 *M*, tube 3, 0·11 *M* urea. After reading, the tubes were left for 15 hours at room temperature. In tube 1 the urea was hypotonic, in tube 3 hypertonic. In tube 2 no increase or decrease took place in the size of the drops, giving a molecular weight of 176. In another experiment a value of 188 was obtained.

Preparation of the phenylosazone. 85 mg. of the sugar were dissolved in 4·5 cc. of water and 0·25 g. phenylhydrazine hydrochloride and 0·55 g. sodium acetate were added. The mixture was heated on a boiling water-bath for half an hour and allowed to cool slowly. The crystals were separated, washed with water, and recrystallised from dilute alcohol. Yield 32 mg. Rapidly heated the melting-point was 208–210°, unchanged when the substance was intimately mixed with a known sample of glucosazone. The optical activity of the product was observed when dissolved in a mixture of 6 parts of pyridine and 4 parts of absolute alcohol. 8·7 mg. in 1·5 g. solvent (*d.* 0·873). $c = 0{\cdot}50$.

Time (hrs.)	0·25	22
α	−0·40	0·22°
$[\alpha]^{13°}_{5461}$	−80	40°

The following figures were obtained on analysis:

C 59·76, 59·92 %; H 6·28, 6·18 %; N 15·57, 15·54 %.

$C_6H_{10}O_4(N : HN.C_6H_5)_2$ requires C 60·33, H 6·14, N 15·64 %.

Preparation of the β-penta-acetate. A mixture of 0·14 g. of the sugar and 0·07 g. of freshly fused sodium acetate was heated with 0·7 cc. of acetic anhydride in a small tube fitted with a reflux condenser. After 2 hours at 100° 4 cc. of cold water were added. The oil which precipitated rapidly crystallised on stirring. The product was washed with several changes of cold water, and three times recrystallised from 90 % ethyl alcohol, in order to remove as much of the α-compound as possible. Yield 34 mg. M.P. 124–125°. $[\alpha]^{13°}_{5461} = +\,5{\cdot}5°$ (in chloroform).

C 48·84, 49·54 %; H 5·77, 5·90 %.

$C_6H_7O_6(OC.CH_3)_5$ requires C 49·23; H 5·64 %.

From a consideration of the experiments described it seems clear that the blood-sugar consists largely of glucopyranose. It has not been possible during the present work to obtain definite evidence as to the presence of any other sugar. The accepted figure for the concentration of reducing substances in normal blood varies from 0·08 to 0·1 %, and the yield of crystalline glucose obtained, taking into account the losses attendant on the various precipitations, does not suggest that more than traces of other sugars can be present; this question is being considered in connection with the metabolism of lactose and will be dealt with in a later communication. In numerous experiments the mother-liquor has been worked up further after crystallisation of the glucose; the syrup obtained has a strongly positive rotation, and gives glucosazone on treatment with phenylhydrazine. The source of the fasting blood-sugar is liver-glycogen. Recent views as to the constitution of this polysaccharide agree that glycogen consists of a conjugated chain of α-glucose units [Haworth, Hirst and Webb, 1929]. When α-glucose is liberated by the action of the liver diastase *in vivo* it seems likely that under the influence of body temperature and blood alkalinity a mixture of the α- and β-stereoisomers will be rapidly established, unless there are some factors involved of which we have no knowledge. Allott [1928] working with rabbits, found no definite difference between the blood-sugar curves after injection of either isomer, or of the equilibrium mixture. The explanation of the low values obtained when the sugar in deproteinised blood-filtrates is estimated by the polarimeter is not yet clear. It has been suggested [Holden, 1926] that glutathione, or ergothioneine [Sjollema, 1927] may be responsible, but the experiments with rabbits after insulin [Winter and Smith, 1924] are difficult to reconcile with this view. When the blood-sugar was removed under the action of insulin and the blood-filtrates examined polarimetrically, a small positive value was always obtained. If any strongly laevo-rotatory substances pass into the Folin-Wu filtrates they should become apparent when the glucose is removed, unless insulin has the effect of diminishing their concentration *pari passu* with the blood-sugar.

SUMMARY.

The blood-sugar of the rabbit has been isolated as α-glucopyranose.

I wish to thank Professor W. N. Haworth for his interest and advice. Part of the expense of this work was defrayed by a grant from the Government Grant Committee of the Royal Society.

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XCIV. THE DISSOCIATION OF CALCIUM CITRATE.

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(*Received May 9th, 1930.*)

It is often stated that the addition of a citrate to milk prevents clotting by rennin because an un-ionised calcium citrate is formed, thus removing the calcium ions from solution, these ions being considered essential for the clotting process. The prevention of the clotting of blood by citrates is explained in the same way.

It is not the purpose of this paper to discuss the clotting phenomenon, but to record some measurements of the dissociation of calcium citrate. However, it must be pointed out that there is some confusion on the subject. A typical analysis of milk-serum [Van Slyke and Bosworth, 1915] shows a concentration of 0·465 g. of calcium per litre and 2·37 g. of citric acid. The citrate is present in a larger proportion than is necessary for equivalence with the calcium, so if un-ionised tricalcium citrate were formed there would be no calcium ions present. Analyses in this laboratory have shown the above figures to be typical of milks which clot normally with rennin. If the theory mentioned in the first paragraph were quite correct, most milks would not clot, for there is normally enough citrate present to prevent this.

Shear and Kramer [1928] and Shear, Kramer and Resnikoff [1929] give results of conductivity titrations of calcium salts with sodium citrate and claim that the results give "direct evidence for the binding of calcium ions by sodium citrate in some kind of soluble complex." They obtained a minimum conductivity when the added sodium citrate was approximately equivalent to the calcium present. As they adjusted the p_H of their solutions with uncertain proportions of acid and alkali, it is impossible to make an estimate of the conductivity of the solution due to any calcium ions present. The only safe deduction from their experiments seems to be that calcium citrate is less ionised than calcium chloride or sodium citrate at the same dilution. Stewart and Percival [1928] state that they have found by conductivity measurements that calcium citrate is almost undissociated in solution, but they give no particulars. It was, therefore, decided to publish the following measurements of the conductivity of simple calcium citrate solutions.

Method and apparatus.

Conductivities were measured by the usual method, using a metre bridge and post-office box with a small induction coil as source of current. The conductivity cell was of 120 cc. capacity and contained electrodes of about 1 cm.^{2} area placed 1 cm. apart. The cell constant was determined by the measurement of the conductivity of $N/10$ potassium chloride at 18° [Reilly, Rae and Wheeler, 1926].

Conductivity water was obtained by redistilling water obtained from a still of the form described by Golding [1906], rejecting the first and last portions of the distillate. It was allowed to come to equilibrium with the atmosphere and usually had a conductivity of about 2×10^{-6} mhos. This was deducted from the values obtained for the conductivity of the solutions measured.

Calcium citrate solution.

This was prepared by mixing concentrated solutions of A.R. calcium chloride and sodium citrate in equivalent proportions. Calcium citrate rapidly precipitated in the high concentration used and was washed repeatedly with distilled water by decantation until 500 cc. of the wash water, when concentrated to 20 cc., gave no reaction for chlorides. The precipitate was then washed a further ten times with conductivity water and finally allowed to stand 2 days in contact with conductivity water in order to prepare the solution for conductivity measurements. This solution was obtained by careful decantation from the solid calcium citrate. All this manipulation was done in one Pyrex flask and the solution stored in Pyrex glass. No attempt was made to obtain a saturated solution of calcium citrate but the difficulties encountered by Shear and Kramer in dissolving calcium citrate were avoided by this procedure.

150 cc. of the solution evaporated to dryness and dried to constant weight at 150° gave 0·1050 g. of total solids containing 0·0251 g. Ca; $Ca_3(C_6H_5O_7)_2$ requires 0·0253 g.

Table I gives results of the measurements of conductivity of different dilutions of this solution at 18°.

Table I.

Temperature 18°.

Normality of $Ca_3(C_6H_5O_7)_2$ solution	Specific conductivity (mhos.)	Equivalent conductivity	Degree of dissociation, α
0·00846	0·000176	20·8	0·188
0·00508	0·000118	23·2	0·209
0·00305	0·0000758	24·8	0·224
0·00183	0·0000498	27·2	0·245
0·00110	0·0000336	30·5	0·275

The degree of dissociation, α, was calculated by taking the values for the mobilities of the Ca^{++} and citrate$^{\equiv}$ ions at 18° as 51 and 60 respectively.

On the assumption that we are simply dealing with the dissociation of $Ca_3(C_6H_5O_7)_2$ it is seen that the dissociation at these high dilutions is small, but by no means negligible (cf. the slight dissociation found by Riley and Fisher [1929] for some metal malonates).

Titration of citric acid with calcium hydroxide.

In order to confirm the above figures and to obtain data for more concentrated solutions, conductivity titrations of citric acid solutions with calcium hydroxide solution were made. As there were no foreign ions present in these solutions it was possible to calculate the dissociation of the calcium citrate formed in the titration.

The citric acid was recrystallised from A.R. grade citric acid, and the calcium hydroxide solution was prepared from lime obtained by heating Kahlbaum's chemically pure calcium carbonate. All solutions were made up using conductivity water.

The results of a titration of 100 cc. of 0·0104 N citric acid with 0·0455 N calcium hydroxide at 18° are shown in Fig. 1. An electrometric titration of the same solutions is also graphed. This was made by using the quinhydrone electrode for p_H measurements. There is a sharp break in the conductivity curve when the calcium hydroxide is present in the proportion necessary for the formation of tricalcium citrate and this end-point is confirmed by the electrometric titration. The volume of calcium hydroxide solution added at the end-point and the conductivity of the resultant calcium citrate solution are readily obtained from the curve, and so the normality of the solution with respect to calcium citrate, and the dissociation, may be calculated.

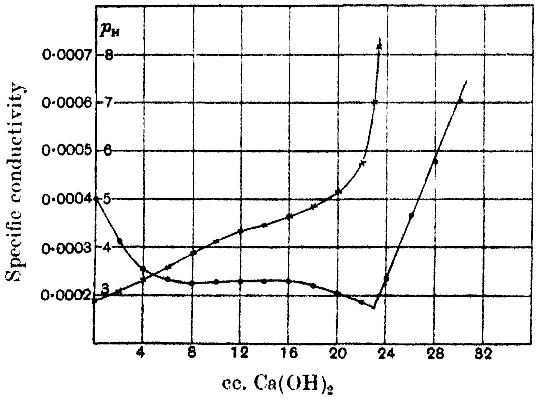

Fig. 1.

•——• Sp. conductivity. ×——× p_H.

Similar titrations were made of various concentrations of citric acid. The most concentrated solution used was 0·0414 N at the end-point. This is comparable to the usual concentration of "citric acid" in milk (about 2 g. per litre). At this concentration the solution is super-saturated. It was found that the conductivity obtained immediately after adding the equivalent proportion of calcium hydroxide did not vary over a period of 5 hours. After standing for about a week calcium citrate had precipitated but the change in conductivity over this period was not investigated.

Table II gives the results of these titrations performed at different dilutions.

Table II.

Temperature 18°.

Normality of $Ca_3(C_6H_5O_7)_2$ at end-point	Specific conductivity at end-point (mhos.)	Equivalent conductivity	Degree of dissociation, α
0·0414	0·000672	16·3	0·147
0·0293	0·000496	16·9	0·152
0·0134	0·000248	18·5	0·166
0·00844	0·000170	20·2	0·183
0·00425	0·000100	23·5	0·212
0·00214	0·000055	25·7	0·232

The values obtained for the degree of dissociation by the two methods are shown in Fig. 2, where they are plotted against normality of the solution.

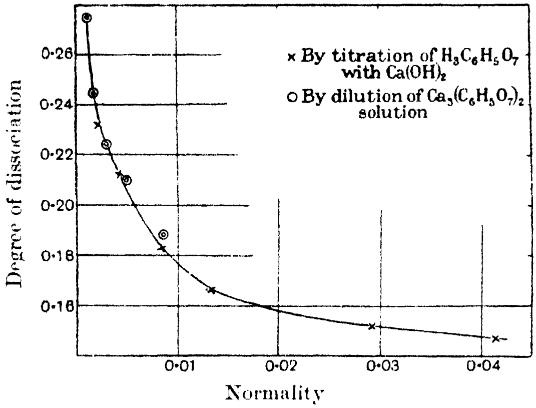

Fig. 2.

There is close agreement between the values. Of course the numerical values obtained for the degree of dissociation could not be transferred directly to such solutions as milk and blood. The effect of ionic concentiation on the dissociation of such a substance as calcium citrate is uncertain. However, the figures give some idea of the order of magnitude of the dissociation.

SUMMARY.

The dissociation of tricalcium citrate in aqueous solution at 18° has been studied, and values for the degree of dissociation are recorded.

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XCV. OBSERVATIONS ON A DIATOM (*NITZSCHIA CLOSTERIUM* W.SM.) AS A SOURCE OF VITAMIN A.

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(*Received May 16th, 1930.*)

INTRODUCTION.

IN 1922 Jameson, Drummond and Coward [1922] studied *Nitzschia closterium*, a typical unicellular marine diatom, and showed that it possessed considerable growth-promoting activity. At that time the fat-soluble vitamins A and D were not differentiated, and these tests, therefore, did not preclude the possibility of the observed growth being due to a factor other than vitamin A. This possibility was indirectly supported by the observation of Drummond and Watson [1922] that the oil from this organism failed to give a positive colour reaction with strong sulphuric acid which Rosenheim and Drummond [1920] had previously shown to be associated with vitamin A. On the other hand, in view of the experiments of Leigh-Clare [1927] it seemed improbable that the growth-promoting activity could be ascribed to vitamin D.

Recently the specificity of the colour reactions of vitamin A with $AsCl_3$ and $SbCl_3$, described by Rosenheim and Drummond [1925] and by Carr and Price [1926], in support of which a weight of evidence has accumulated during the last few years, has been questioned by various investigators. The discrepancies have been satisfactorily explained by Drummond and Morton [1929] and by Ahmad and Drummond [1930], but the controversy recalled the earlier observations of Drummond and Watson [1922] regarding the oil from *Nitzschia closterium* which, though apparently a potent source of vitamin A, did not give the colour reaction. It was decided, therefore, to repeat the experiments, and also get a more quantitative estimate of the activity of this organism in the light of our present knowledge of the subject.

EXPERIMENTAL.

Following mainly the technique of Allen and Nelson [1910], large quantities of the organism were grown from a pure culture obtained by the courtesy of Dr E. J. Allen, F.R.S., Director of the Marine Biological Laboratory, Plymouth. 100 cc. of sea water, to which Miquel's nutrient solutions, as modified by Allen, had been added, were placed into each of a large number of conical

Pyrex flasks of 250 cc. capacity, plugged with cotton-wool and sterilised in steam at 95° for 20 minutes on three successive days. Each flask was then inoculated with 0·01 cc. of the pure culture and placed in front of a window in diffused daylight. The bottoms of the flasks were soon covered with a brown growth of the organism.

A large quantity of the organism being required, cultures and subcultures were continued for a period extending over a year. Cultures usually stopped growing after 4–6 weeks. During the winter months when the daylight was poor the growth was scanty.

Four to six weeks-old cultures were filtered on a tared filter, dried in a vacuum desiccator and weighed. They were stored in a desiccator, which had been re-evacuated after the air had been replaced by nitrogen, in a cool, dark cupboard. The oil was extracted with redistilled ether by continuous extraction in Soxhlet's apparatus. Nitrogen was passed in during the extraction and other precautions were taken to prevent oxidation.

The results of culture experiments are summarised in Table I.

Table I.

Crop	Time of year	Vol. of the culture sol. (litres)	Dry wt. of organism (g.)	Quantity of oil obtained (g.)
1	Apr. and May	3·7	3·55	0·103
2	June, July and Aug.	12·5	11·74	—
3 (*a*)	Sept. and Oct.	5·5	5·53	0·156
(*b*)	Sept. and Oct.	3·0	1·10	—
4	Oct., Nov. and Dec.	15·5	9·075	0·374
5	Dec., Jan. and Feb.	15·0	2·765	

Under satisfactory conditions of growth approximately 1 g. of the dry organism was obtained from each litre of a month-old culture. During the dark winter days the yield fell even below 0·2 g. per litre. The amount of the oil in the dry organism was, however, fairly constant and varied between 2·8 and 3·1 %.

The oil is extremely rich in natural plant pigments, chlorophyll, xanthophyll, carotene, and fucoxanthin, the characteristic pigment of the brown algae. It gave a strong blue colour reaction with $SbCl_3$. Drummond and Watson failed to get the colour reaction with H_2SO_4, probably because they had rather a small amount of the material available, and because with this reagent the test is not very delicate.

Table II shows the yellow and blue colour units given by 10 % solutions in a 1 cm. cell and examined in the Rosenheim-Schuster colorimeter. In the case of blue units the figures refer to 0·2 cc. of 10 % oil mixed with 2 cc. of $SbCl_3$ reagent, and examined after 30 seconds. The technique of Carr and Price [1926] was mainly followed, though 10 % values were obtained from an intensity-dilution curve as explained in our previous communication [Ahmad and Drummond, 1930].

The pigment of *Nitzschia closterium* interferes with the application of the $SbCl_3$ reaction. Attempts have been made to obtain the non-saponifiable fraction of the oil in order to remove chlorophyll, but sufficient material was not available. For this reason the measurement of the blue colour must be regarded as approximate.

Table II.

Crop	Dilution %	Colour units 1 cm. cell	Blue units with $SbCl_3$ reagent
1	10	3,000 yellow 429 red	57·1 blue 52·4 yellow
3 (*a*)	10	10,000 yellow 830 red	100–150 blue 100–120 yellow
4, 5	10	10,000 yellow 800 red	100–150 blue 100–120 yellow

The technique of the biological test was that usually employed in this laboratory [see Drummond and Morton, 1929]. The basal diet consisted of purified caseinogen, rice starch and salt mixture without the addition of any extraneous fat. Vitamin D was supplied as irradiated ergosterol dissolved in medicinal liquid paraffin, and 0·01 mg. was given twice a week to each rat. Ethyl laurate was used as a diluent for the oil. Its satisfactory nature has been shown by Drummond, Ahmad and Morton [1930].

For feeding, the filter-paper with the organism was broken up into small pieces, mixed with a small quantity of water and the basal diet, and worked up into a dough. This was given to the rats in aliquot portions on small porcelain dishes. They devoured it readily.

The results of the biological tests are shown in Table III, and Figs. 1–3 show some representative curves.

Table III.

No. and sex of rat	Dose and its nature	Total days of test	Total difference in wt. (g.)	Rate of growth per week (g.)
27 ♂	0·2 mg. oil	16	− 3	− 1·3
26 ♀	0·4 mg. oil	16	0	0
28 ♂	1·0 mg. oil	16	− 11	− 4·8
69 ♂	0·35 g. organism	21	+ 45	+ 15·0
70 ♀	0·35 g. organism	21	+ 31	+ 10·0
72 ♀	0·1 g. organism	21	+ 27	+ 9·0
95 ♀	5·0 mg. oil	14	+ 13	+ 6·5
362 ♀	1 mg. oil	7	0	0
	5 mg. oil	14	+ 15	+ 7·5
385 ♂	2 mg. oil	11	− 5	− 3·2
	0·15 g. extracted organism	7	− 3	− 3·0
	0·15 g. extracted organism plus 5 mg. oil	14	+ 30	+ 15·0
388 ♀	3 mg. oil	11	+ 2	+ 1·2
	5 mg. oil	14	+ 15	+ 7·5

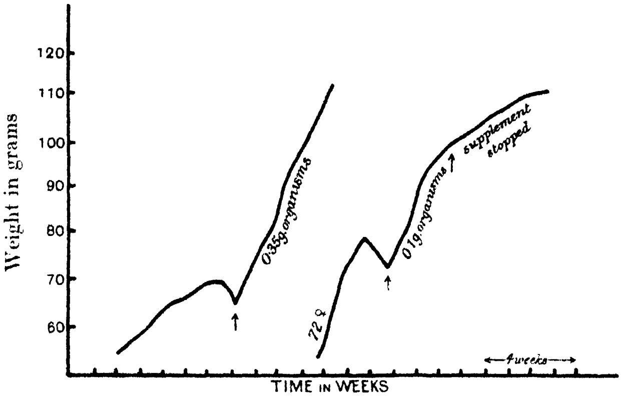

Fig. 1. Administration of dried organism.

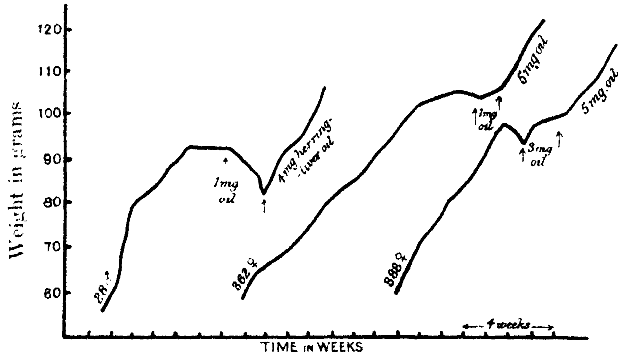

Fig. 2. Administration of oil.

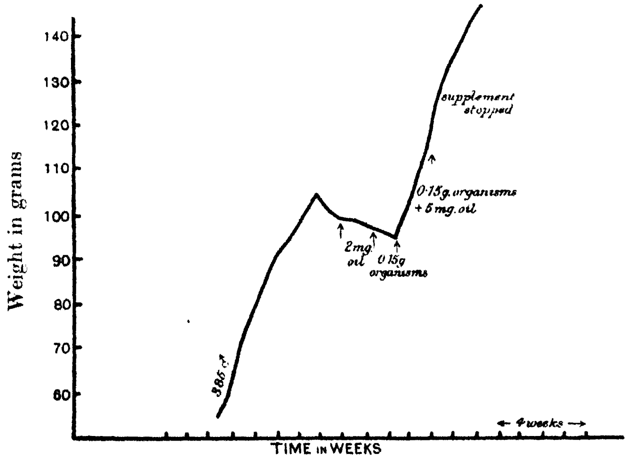

Fig. 3. Administration of extracted organism plus oil.

DISCUSSION.

The feeding experiments conclusively indicate that *Nitzschia* and the oil obtained from it are good sources of vitamin A. The extracted oil gives a positive reaction with $SbCl_3$ and the apparent discrepancy observed by Drummond and Watson does not exist.

The extreme richness of the oil in the natural plant pigments will be clear from a study of Table II. The greater part of the colour is due to chlorophyll and xanthophyll, both of which are inactive as a source of vitamin A [Willimott and Moore, 1927]. Carotene, whose relation to vitamin A is supported by increasing evidence, is also present, and it is possible that the greater part of the physiological activity of the oil is due to this pigment. A spectroscopic examination would have indicated the part of the activity due to carotene and the part due to vitamin A, but unfortunately enough material was not available for this to be made. If we compare the biological activities of this pigmented oil and carotene (rat dose 5 mg. and 0·005 mg. respectively) and their respective yellow colours (10,000 and 300,000 Lovibond units in 10 % dilution in 1 cm. cell), we find that the whole of the biological activity of the oil can be accounted for if 3 % of the yellow pigment present be carotene. This is interesting when considered with the earlier observations of Steenbock and his colleagues pointing to the association of the lipochrome pigments and vitamin A activity of vegetable sources; of Coward [1923] that the lipochrome chiefly concerned in this association was carotene; the more recent work on the activity of carotene; and our own observations and that of Moore [1929, 1930] that from the livers of rats fed on large quantities of carotene (0·02–0·05 mg. per day) a colourless or a lightly coloured fat is obtained which gives an intense colour reaction with $SbCl_3$. It will probably not be far from the truth if it is suggested that these diatoms are the ultimate source of vitamin A of the fish-liver oils, and that a similar change takes place in the cod or the intermediate fauna giving rise to the stores of the colourless vitamin A present in the liver oils. It also explains why liver oils of fish may be very pale in colour and at the same time very rich in vitamin A, a fact which had rather perplexed the earlier supporters of the view that vitamin A was related to a lipochrome pigment.

SUMMARY.

1. The oil obtained from a marine diatom (*Nitzschia closterium*) growing in pure culture, has been studied both colorimetrically and biologically as a source of vitamin A.

2. It has been found that the oil is very potent in this respect and gives a strong colour reaction with $SbCl_3$.

3. The greater part of the biological activity of the oil is probably due to carotene, synthesised by the organism, and extracted with the oil.

In conclusion, I desire to thank Prof. J. C. Drummond for his kind help and advice throughout the course of this investigation, and the Medical Research Council for a grant in aid of the expenses.

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XCVI. THE COMPOSITION OF THE FATTY ACIDS PRESENT AS GLYCERIDES IN THE LIVER OIL OF THE THRESHER SHARK (*ALOPOECIA VULPES*).

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(*Received May 27th, 1930.*)

In a recent communication [Guha, Hilditch and Lovern, 1930] an account was given of quantitative analyses of the fatty acids of a series of fish-liver oils. When these were subsequently arranged according to their biological families, the oils of the elasmobranch group, including shark, dogfish and skate, lent support to the view that there was a definite and close relation between the amount of squalene present in the non-glyceride portion of the oil and the nature of the fatty acid constituents. When the amount of squalene was high, as in *Scymnorhinus lichia*, the acids were almost exclusively monoethylenic, and contained, in contrast to other fish-liver oils examined, such as those of the Teleostomi group, appreciable amounts of an unsaturated acid containing twenty-four carbon atoms (selacholeic acid). As the percentage of squalene present decreased there was a steady increase in the degree of unsaturation of the fatty acids, together with a steady diminution in the amount of C_{24} acid present, *e.g. Squalus acanthias*, with *ca.* 4 % of squalene (10·5 % unsaponifiable). In the liver oil of the skate (*Raia maculata*) studied, squalene was practically entirely absent, the unsaturation of the C_{20} and C_{22} groups of acids was exceedingly high, and acids of the C_{24} group were not present. It is highly desirable that further information regarding the liver oils of members of this group should be obtained, since the correlation outlined above rests at present on but a few instances.

In this connection the author was fortunate to obtain access to the fatty acid portion of the liver oil of a thresher shark (*Alopoecia vulpes*), the liver of which had been secured by Prof. J. C. Drummond of University College, London, to whom, with Mr L. C. Baker, the writer desires to express his thanks for the information given regarding the crude oil. This particular shark was caught off the Dorset coast on the 18th of June, 1928, and landed at Abbotsbury. The weight of the fish was 4 cwt., and the liver, procured on the 20th of June, weighed 4 kg. The final yield of oil (prepared by steaming) was 650 g. This was of a golden brown colour, with some separation of crystalline matter on standing. The characteristics of the oil were as follows:

% free fatty acids (as oleic)	0·20
Saponification value	181
Iodine value (Dam)	176
Refractive index 20°	1·4741
% unsaponifiable	1·83

The iodine value of the unsaponifiable matter was 102 and it contained 21·8 % of cholesterol; this corresponds with an iodine value for the rest of the unsaponifiable matter of about 112, as against 370 for squalene, so that the amount of squalene (if any) in thresher shark-liver oil must be very low. From this standpoint it is therefore in the same category as skate-liver oil (*Raia maculata*), and, as the following analysis shows, the fatty acid composition is also remarkably similar to that of skate-liver oil. The method of analysis was precisely the same as that given for the other oils [Guha *et al.*, 1930] and only the actual data are given here. It may be noted in passing that in both skate- and thresher shark-liver oils there was very little tendency to polymerisation, although both oils had been kept for over a year before analysis, and both contained a large proportion of acids of a very high degree of unsaturation.

EXPERIMENTAL.

Fatty acids; I.V. 181·4, sap. eq. 289·6.

Lead salt separation.

	g.	%	I.V. (of corresponding methyl esters)
"Solid" acids S	51·0	24·5	38·64
"Liquid" acids L	149·0	74·5	236·3

Fractionation of methyl esters.

Primary fractions					Refractionations				
No.	g.	B.P./1 mm. °C.	S.E.	I.V.	No.	g.	B.P./1 mm. °C.	S.E.	I.V.
(i) *Esters of "solid" acids S* (49·5 g.).									
S 1	35·37	100–130	261·0	10·69					
S 2	5·73	130–145	289·6	73·99					
S 3	8·00	Residue	325·8	143·2					
(Fraction S 1 (16·72 g.) gave 15·00 g. of saturated esters (S.E. 259·4) on oxidation.)									
(ii) *Esters of "liquid" acids L* (145 g.).									
L 1	39·79	127–140	272·1	113·6	L 11	8·49	105–122	249·7	68·0
					L 12	18·92	122–128	270·4	104·4
					L 13	8·07	Residue	292·1	188·8
L 2	34·36	140–158	306·5	218·6	L 21	4·42	120–139	290·6	148·5
					L 22	19·09	139–143	304·5	204·4
					L 23	6·27	Residue	319·6	286·9
L 3	56·44	158–175	329·6	320·9	L 31	4·20	132–165	307·3	276·4
					L 32	29·54	165–170	327·3	320·3
					L 33	10·15	170–175	331·8	342·2
					L 34	5·66	Residue	331·1	326·5
L 4	13·83	Residue	346·3*	290·2					

* Saponification equivalent of residual esters, freed from unsaponifiable matter, L 4 : 337·7.

Estimated composition of fatty acids.

	"Solid" acids S (24·5 %)	"Liquid" acids L (74·5 %)	Total %	Fatty acids	
				%	Mean unsaturation
Saturated					
Myristic	5·8	1·6	7·4	7·4	
Palmitic	11·3	—	11·3	11·3	
Stearic	0·2	—	0·2	0·2	
Unsaturated					
C_{14} group	—	1·6	1·6	1·6	
C_{16} ,,	—	12·0	12·0	12·0	(– 2·0 H)
C_{18} ,,	4·0	15·1	19·1	19·2	(– 3·4 H)
C_{20} ,,	3·7	27·2	30·9	31·0	(– 6·6 H)
C_{22} ,,	0·5	16·8	17·3	17·3	(– 10·5 H)
Unsaponifiable	—	0·2	0·2	—	

It will be observed that the results for thresher shark-liver oil are in entire agreement with the theory already tentatively advanced, but it should be reiterated that the number of cases examined up to the present is insufficient for this generalisation to be regarded as proved. More data are evidently very desirable before any deductions are seriously advanced, but some reference to the many problems suggested by such a relation (if proved) may perhaps be permitted. For instance, is the relation between the squalene content and the fatty acids definite for any particular species? Only one sample of oil from each species has been fully examined, whereas it is known that the unsaponifiable content of particular members of the Elasmobranchi may vary between wide limits. Thus, unsaponifiable matter between the limits of 4 and 32·9 % has been found in the liver oil of the dogfish (*Squalus acanthias*), a mixture of squalene and cholesterol being present in many cases [Grün and Halden, 1929]; liver oils of the basking shark (*Cetorhinus maxima*) have been observed [Grün and Halden, 1929] to contain from 20 % to as much as 56 % of unsaponifiable matter (of which approximately one-half is frequently squalene); whilst, similarly, in oils from livers of *Scymnorhinus lichia* [Channon, 1928] the unsaponifiable content varied from 48·5 % to 81·5 %.

There is thus no reason to assume that skate-liver oil, for instance, will always be found to contain negligible amounts of squalene. Channon [1928] has given evidence which suggests that the squalene present in these fish-liver oils is not taken in the food, but is synthesised by the fish. To mention two results: (1) there is no squalene present in the various samples of plankton examined; (2) the feeding habits of all members of the Squalidae family appear to be similar, and yet squalene is present in large amount in some, and entirely absent from others.

One has also to take into account, if accepting the theory of synthetic origin, the fact that squalene is by no means the only unsaponifiable matter present in elasmobranch oils. Other substances frequently found (in varying amounts, sometimes alone and sometimes with squalene) include hydrocarbons (*e.g. iso*octadecane, decane, etc.), cholesterol, and ethers such as batyl, chimyl and selachyl alcohols. It has yet to be determined whether there is any relation between the presence or absence of these substances and the composition of the accompanying fatty acids.

In conclusion, the author wishes to express his thanks and appreciation to Prof. T. P. Hilditch for his kind interest and advice throughout the course of this work.

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XCVII. VITAMIN A OF BUTTER.

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(*Received May 10th, 1930.*)

It is now clear that the physiological action of the vitamin A of liver fats can be reproduced, or at any rate closely simulated, by the plant pigment carotene [Euler, Euler and Hellström, 1928; Euler, Euler and Karrer, 1929; Moore, 1929, 1, 2; 1930; Collison, Hume, Smedley-MacLean and Smith, 1929], and it therefore becomes important to ascertain

(*a*) whether butter contains either or both of these substances;

(*b*) the relative importance of these substances, if both are present;

(*c*) whether the quantities of either or both vary with seasonal, dietary or other conditions.

In the present paper an account is given of a preliminary survey of this question wherein we have applied spectrographic methods as a means of arriving at a decision concerning the above points.

Before dealing with the absorption spectra of the butter extracts themselves it is necessary to recall that both carotene and the vitamin A of liver fats can be recognised in extracts of non-saponifiable matter by their absorption spectra and also by the spectral location of the principal absorption band in the blue solutions obtained by the well-known antimony trichloride reaction.

Absorption spectra.

A solution of carotene in chloroform shows maxima at 492, 462, 436, 348 and 279 $\mu\mu$, whilst in alcohol the visible bands appear at 480, 451 and 427 $\mu\mu$. Whereas the ultra-violet absorption of carotene is weak relatively to the visible absorption [Dulière, Morton and Drummond, 1929], liver oils and non-saponifiable extracts on the other hand show negligible absorption in the visible, but in the ultra-violet a strong band with a maximum near 328 $\mu\mu$, the intensity of absorption at the head of the band affording a trustworthy measure of the vitamin A potency [Morton and Heilbron, 1928, 1930].

The non-saponifiable matter from a sample of New Zealand butter exhibited clear maxima at 492, 462 and 432 $\mu\mu$ when the solvent was chloroform and at 480, 452 and 427 $\mu\mu$ when alcohol was used. Comparison of the intensity of absorption for pure carotene and for the butter extracts indicated that the latter contained about 1 part in 200 of the pigment. The butter extract is, however, sharply differentiated from the absorption spectrum of carotene itself by the fact that it exhibits rapidly increasing intensity of absorption on the ultra-violet side of 380 $\mu\mu$ (Fig. 1). The absorption curve shows a marked inflexion at 310–340 $\mu\mu$, and there can be little doubt that the substance responsible for this is identical with the vitamin A of liver oils. In addition to this, however, another substance which shows a clear maximum in the neighbourhood of 272–275 $\mu\mu$ (Fig. 3) is also present in the butter extract. The observed ultra-violet absorption of the butter extract is therefore a summation of three separate effects:

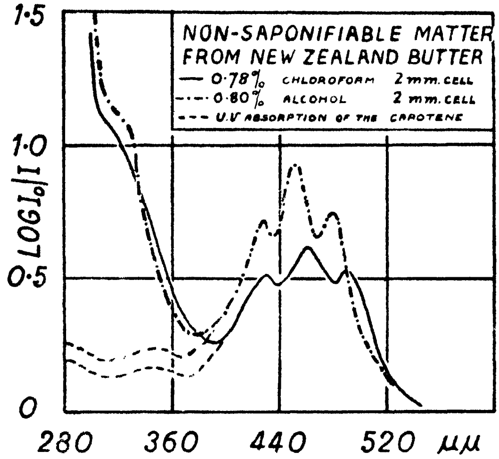

Fig. 1.

(1) the ultra-violet absorption of the carotene, the intensity being deducible from the visible absorption of the butter extract by utilising the known absorption curve for pure carotene;

(2) the ultra-violet absorption of the unidentified substance characterised by a maximum near 272 $\mu\mu$;

(3) the ultra-violet absorption of the distinct vitamin A which has hitherto been associated mainly with fish-liver oils.

The impossibility of distinguishing quantitatively between the two types of ultra-violet absorption restricts the interpretation of the absorption spectrum of butter extracts in the ultra-violet to fixing an upper limit to the potency of the vitamin A. Calculations indicate that the non-saponifiable matter from the New Zealand butter contains, in addition to about 0·5 % of carotene, sufficient vitamin A to make its potency not more than about six times that of a good cod-liver oil[1].

An examination of the non-saponifiable matter of Danish butter (commercial sample) provides evidence of a much smaller carotene content (Fig. 2), this being of the order of 0·1–0·15 %; as regards the vitamin A content, the upper limit does not appear to be very different from that for the New Zealand butter. On the other hand the concentration of the material responsible for the band near 272 $\mu\mu$ is very definitely greater (Fig. 3), and this last observation would tend to show that the true vitamin A content of the Danish butter is lower than that of the New Zealand butter, the high concentration of the unidentified substance being probably responsible to some extent for the intensity of absorption near 320 $\mu\mu$.

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[1] This figure is arrived at by comparing the intensities of absorption at 328 $\mu\mu$ for equal concentrations and all thicknesses of cod-liver oil and butter concentrate.

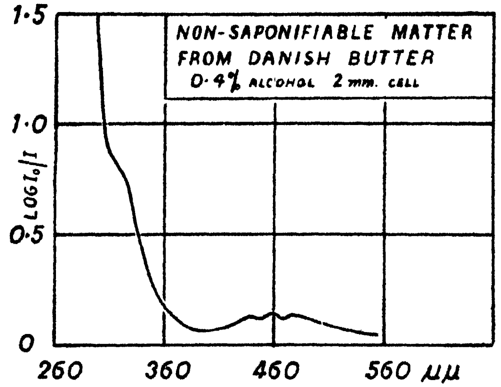

Fig. 2.

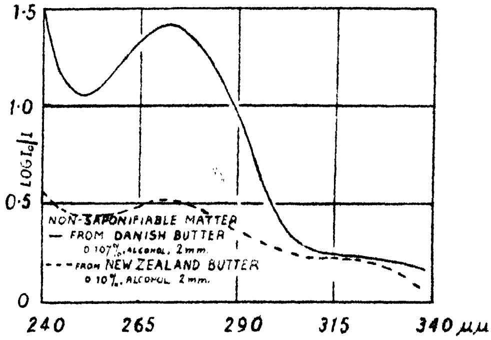

Fig. 3.

Antimony trichloride reaction.

The blue colour obtained with pure carotene is characterised by a well-defined band at 591 $\mu\mu$ [Dulière, Morton and Drummond, 1929], whilst that obtained with non-saponifiable matter from liver oils shows a sharp maximum at 624 $\mu\mu$ [Morton and Heilbron, 1928, 1930]. Using the New Zealand extract both these maxima are shown clearly at 624 and 592 $\mu\mu$. The intensity of the 624 $\mu\mu$ band was about five times that associated with an equal weight of a good cod-liver oil, whilst the less intense band at 592 $\mu\mu$ indicated a maximal carotene content of 2 %. This figure is undoubtedly too high, as the liver oil concentrates (in which carotene is certainly not present in any significant amounts) often give high, if somewhat unselective, absorption at 592 $\mu\mu$.

The non-saponifiable matter from Danish butter gave a good colour with antimony trichloride, the tint being definitely more green than blue. A very sharp band at 620–622 μμ was seen but no band at 592 μμ could be recognised. This was not surprising as the spectrographic data had already proved that the carotene content was relatively low. The intensity of the 620–622 μμ band indicated that the potency of the Danish butter extract was about 2·45 times that of cod-liver oil.

It will thus be seen that the direct absorption spectra data and the study of the antimony trichloride blue colours give a consistent account of the properties of the non-saponifiable matter from butter.

The recognition of carotene together with the distinct vitamin A of liver fats in a product derived from milk is of importance and lends support to Moore's view [1930] that carotene can be converted *in vivo* to vitamin A.

It should be emphasised that the butters used were not chosen as representative of their respective classes, and many more samples will need to be studied before any generalisations can be made.

We are indebted to Messrs Joseph Nathan and Co., Ltd. for the preparation of the butter extracts and to the Department for Scientific and Industrial Research for a grant which has enabled this investigation to be carried out.

SUMMARY.

The non-saponifiable matter from butter has been shown spectroscopically to contain carotene, vitamin A, and an unidentified substance characterised by selective absorption at 272 μμ. The carotene and vitamin A potency can be estimated with some degree of accuracy.

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XCVIII. THE ACTION OF THE CINCHONA AND CERTAIN OTHER ALKALOIDS IN BIRD MALARIA.

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(*Received May 27th, 1930.*)

Although cinchona bark first appeared in Europe about 1639 and the first cinchona alkaloid, quinine, was isolated from it in 1820 and has been manufactured and used in medicine ever since, there are still many unsolved problems in connection with the use of cinchona alkaloids in malaria.

Some of these questions are of great practical importance. Thus it has been assumed that for the treatment of malaria the best of the four principal cinchona alkaloids is quinine, and over 90 % of the bark now produced from cinchona plantations is bark cultivated to give high yields of quinine.

In the last few years misgivings have arisen as to whether this is the best policy to adopt, and it has even been suggested that it might be advisable to go back to the plan adopted in British India and Ceylon in the early days of cinchona planting and to cultivate the hardy *Cinchona succirubra* species, which yields a mixture of alkaloids, in which quinine and cinchonidine predominate, and to prepare this mixture as "quinetum" for the large scale treatment of malaria [Gage, 1925]. As a basis for the serious discussion of such questions it is desirable that the relative therapeutic values of the principal crystalline alkaloids found in cinchona bark, viz. quinine, quinidine, cinchonidine and cinchonine, should be determined. Hitherto, the only method of making such a comparison has been by clinical trials. The difficulties of making such comparative trials are explained in a report by the Medical Research Council [1925] dealing with the relative values of quinine and quinidine. Probably the difficulties experienced in the case of these two bases have so far precluded extension of the investigation to a similar comparison between quinine and the other readily procurable cinchona alkaloids, cinchonidine, cinchonine, and the reduction product of quinine, hydroquinine. If, in such a comparative trial, cinchonidine proved to be as useful as quinine, there would be a *prima facie* case for the use of "quinetum" in place of quinine.

The relative values of the four alkaloids are also of importance in connection with the use of "cinchona febrifuge," which consists of the total residual alkaloids of cinchona bark, left after the removal of as much of the crystallisable alkaloids as manufacturers normally require to meet commercial demands. Large quantities of this material are now used in malarial countries and it is, as might be expected, of varying quality [Howard, 1925]. Its use has numerous advocates, but before this can be placed on a scientific basis, a satisfactory method of analysis for cinchona febrifuge is required so that definite standards can be set up for it [Goodson and Henry, 1930]. These standards ought to take into account the relative therapeutic values of the components as well as industrial convenience.

It is also sometimes suggested that the special value attributed by some authorities to the antimalarial activity of cinchona febrifuge is due to the presence of some highly active and so far undiscovered component [Prain, 1925].

Apart from these practical problems, there are also questions of purely scientific interest. Much discussion has taken place as to the particular part of the complex structure of the quinine molecule which determines its antimalarial activity, and certain tentative conclusions have been reached based partly on *in vitro* tests with protozoa and partly on limited clinical trials, but there is still no certainty about these conclusions, though they may have an important bearing on the synthesis of new antimalarial drugs [Kaufmann, 1926; Giemsa, Weise and Tropp, 1926; Shaw, 1928].

Further, in the course of the last 10 years many "modified cinchona alkaloids" have been prepared in the laboratory and for some of these claims have been made for increased action in malaria.

It seemed to us that the technique standardised by Roehl [1926] for testing the efficacy of drugs in bird malaria might be used with advantage to investigate some of these questions since it was by this means that the efficacy of "plasmoquin" in malaria was discovered, though the case of "dimeplasmin" [Green, 1929], which was investigated in the first instance by the same method, indicates that positive results in bird malaria may not always indicate that a drug will be efficacious in human malaria.

Among the "modified cinchona alkaloids" the series of "quitenines" obtained by the gentle oxidation of the four principal cinchona alkaloids, whereby the $CH : CH_2$ group in the quinuclidine nucleus (R' in the generalised formula, p. 876) is oxidised to a carboxyl group, occurred to us as a promising basis for a homologous series of products, the "alkylquitenines," in which the value of Roehl's technique for dealing with questions of this kind could be tested. It is, for example, well known that in *in vitro* tests of bactericidal agents action increases as a homologous series is ascended up to a maximum and then diminishes. In the series of 4-*n*-alkylresorcinols, maximal effect is reached at hexylresorcinol [Johnson and Lane, 1921; Dohme, Cox and Miller, 1926], in the *p*-*n*-alkylphenols at *n*-amylphenol [Coulthard, Marshall and Pyman, 1930] and in the alkyldihydrocupreines at octyldihydrocupreine [Schäffer, 1917; Bieling, 1918]. But this principle, though it is traceable in the results of a number of investigations [*e.g.* Morgenroth and Tugendreich, 1916], has not been established with the same certainty in *in vivo* therapeutic tests. It has, however, already been shown that in a short series of cupreine ethers the following changes in activity in bird malaria occur. The figures in brackets give the retardation of attack in days [Giemsa, Weise and Tropp, 1926]:

Cupreine (0–4).

Cupreine ethers:

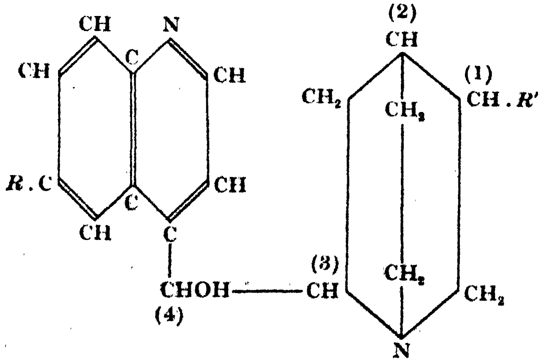
Methyl: quinine (12–13); quinidine (12).
Ethyl: quinethyline (8–40).
Propyl: quinpropyline (4–10).
Amyl: quinamyline (7–11).

With regard to quitenine itself it has been clearly established that this substance is inactive in both human and bird malaria, but that activity is recovered in the ethyl ester [Giemsa, 1926]; investigation in this series does not seem to have been carried beyond this single observation.

Finally, there is a considerable number of natural drugs other than cinchona bark and its constituent alkaloids which have local reputations as remedies for malaria or as febrifuges. Some of these, such as the *Alstonia* barks, have been recognised in Pharmacopoeias, but the evidence for their therapeutic value is slender. The discovery of a highly active substance among them might provide a new starting-point for the synthesis of antimalarial drugs, and it seemed reasonable to suppose that Roehl's method might at least enable any promising product to be picked out of this group for further examination. The present paper records the results of attempts to apply this method to the solution of some of these problems.

General formula for cinchona alkaloids.

As it will frequently be necessary to refer to derivatives of the various cinchona alkaloids, it may be convenient at this stage to give the following generalised formula for the principal cinchona alkaloids:



R = H in cinchonine, cinchotenine, dihydrocinchonine, cinchonidine, cinchotenidine and dihydrocinchonidine: in all other cases it is $\cdot OCH_3$, except in cupreine where it is $\cdot OH$ and the cupreine alkyl ethers, quinethyline ($\cdot OC_2H_5$); quinpropyline ($\cdot OC_3H_7$); quinamyline ($\cdot OC_5H_{11}$).

R′ = $\cdot CH:CH_2$ in cinchonine, cinchonidine, quinine and quinidine

= $\cdot CH_2.CH_3$ in the dihydro-reduction products of these four alkaloids

= COOAlk. for the alkyl-quitenines, quitenidines, cinchotenines and cinchotenidines; Alk. = methyl, ethyl, propyl, etc.

Carbon atoms 1, 2, 3, 4 are all dextrorotatory in cinchonine and its derivatives and in quinidine and its derivatives: carbon atoms 1 and 2 are dextrorotatory and 3 and 4 laevorotatory in cinchonidine and quinine and their derivatives [King, 1922].

PREPARATION OF THE DRUGS.

The cinchona alkaloids used were laboratory stock, which had been used for other investigations and were of satisfactory purity. The hydrogenated alkaloids were prepared from them by reduction with hydrogen in presence of palladium as a catalyst. The esters of quitenine and of the analogous acids derived from cinchonidine, quinidine and cinchonine were prepared by the general method described below. Many of them are new and consequently it is desirable to include a brief record of their chemical characteristics: this is given below, the names of the new substances being printed in italics.

The *iso*quinoline alkaloids referred to on p. 888 were laboratory specimens prepared mainly by Dr F. L. Pyman, F.R.S., in the course of former investigations carried out in one of these laboratories.

The other preparations referred to on pp. 887, 888 are mainly derived from chemical investigations now in progress. The various "total alkaloids as hydrochlorides" were prepared by extracting the total alkaloids of the plant specified and neutralising these with hydrochloric acid to form a neutral solution of known strength. This rule of using all the drugs as neutral hydrochlorides was only diverged from when the neutral hydrochloride was too sparingly soluble to enable a dose completely in solution in 0·5 cc. to be given, and in such cases either the acid hydrochloride or a still more soluble salt, such as the acetate or the ethylsulphonate, was used.

Esters of quitenine, quitenidine, cinchotenine, cinchotenidine.

The esterifications were effected with good yields, in the manner described for methylquitenine, unless otherwise stated.

Methylquitenine. Dry hydrogen chloride was passed into a mixture of anhydrous quitenine (10 g.) and methyl alcohol (50 cc.) heated on the water-bath for 2 hours. After removal of methyl alcohol by distillation, the aqueous solution of the residue was made alkaline with sodium carbonate solution, and the methyl ester extracted by chloroform, the small amount of unchanged quitenine, which separated, being removed by filtration.

The base was not obtained crystalline. It softened at 165° (corr.), melted at 170° (corr.) and had $[\alpha]_D^{20°}$ – 130° ($c = 2{\cdot}24$ in chloroform). (Found: C, 67·1; H, 6·9. $C_{20}H_{28}O_4N_2$ requires C, 67·4; H, 6·8 %.) *Methylquitenine monohydrochloride* was prepared by neutralising a solution of the base in alcohol with dilute hydrochloric acid. It crystallised in plates (frothing at 85°) with difficulty from methyl alcohol on adding dry ether, and once crystallised it could readily be recrystallised from benzene in needles, frothing at 96°. The anhydrous salt commenced to change at 153° (corr.) and gradually frothed. It had $[\alpha]_D^{21°}$ – 189° ($c = 0{\cdot}994$ in water) and was very soluble in water. (Found in air-dried crystals from methyl alcohol-ether: loss at 105°[1] in a vacuum, 7·4. $C_{20}H_{24}O_4N_2$, HCl, $2H_2O$ requires H_2O, 8·4 %. Found in dry salt: C, 60·9; H, 6·8; Cl, 8·8; MeO, 15·6. $C_{20}H_{24}O_4N_2$, HCl requires C, 61·1; H, 6·4; Cl, 9·0; 2MeO, 15·8 %.)

Ethylquitenine crystallises from wet ether or alcohol, or better from a mixture of chloroform and ethyl acetate, in anhydrous needles, M.P. 201° (corr.), and has $[\alpha]_D^{20°}$ – 141° ($c = 1{\cdot}5935$ in chloroform). (Found: C, 67·9; H, 7·6. $C_{21}H_{26}O_4N_2$ requires C, 68·1; H, 7·1 %.) Ethylquitenine monohydrochloride was recrystallised from acetone. The dry salt sintered at 158° (corr.), melted at 162° (corr.) and had $[\alpha]_D^{16°}$ – 206° ($c = 0{\cdot}988$ in water). It is very soluble in water. (Found: Cl, 8·6. $C_{21}H_{26}O_4N_2$, HCl requires Cl, 8·7 %.) Ethylquitenine dihydrochloride crystallises from alcohol. It is hygroscopic and very soluble in water. (Found in dry salt: Cl, 15·7. $C_{21}H_{26}O_4N_2$, 2HCl requires Cl, 16·0 %.)

n-*Propylquitenine* separated from acetone in anhydrous crystals, apparently tetrahedra, melting at 170° (corr.) and having $[\alpha]_D^{17°}$ – 143° ($c = 2{\cdot}048$ in chloroform). (Found: C, 68·8; H, 7·6. $C_{22}H_{28}O_4N_2$ requires C, 68·7; H, 7·3 %.) n-*Propylquitenine monohydrochloride* crystallised from benzene in needles, frothing at 89°. (Found in air-dried salt: loss at 100° in a vacuum, 8·4. $C_{22}H_{28}O_4N_2$, HCl, $2H_2O$ requires H_2O, 7·9 %.) The anhydrous salt sintered at 125° (corr.), melted at 187° (corr.) and had $[\alpha]_D^{20°}$ – 225° ($c = 1{\cdot}01$ in water). Solubility 1 in 7 of water at 23°. (Found: Cl, 8·3. $C_{22}H_{28}O_4N_2$, HCl requires Cl, 8·4 %.)

iso*Propylquitenine* crystallised from benzene in anhydrous clusters of plates, sintering at 184° (corr.), melting at 189° (corr.) and having $[\alpha]_D^{22°}$ – 142° ($c = 2{\cdot}011$ in chloroform). (Found: C, 68·8; H, 7·6. $C_{22}H_{28}O_4N_2$ requires C, 68·7; H, 7·3 %.) iso*Propylquitenine monohydrochloride* crystallised from benzene in needles, frothing at 88° (corr.). (Found in air-dried salt: loss at 105°

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[1] When the air-dried substances described in this paper underwent change below 105°, they were dried first at a low temperature and finally at the temperature recorded. In a number of cases the salts crystallised with benzene, which was partly replaced by water on exposure to air, the products becoming sticky. In these cases the loss on drying does not correspond with any definite number of molecules of either solvent.

in a vacuum, 11·5 %.) The anhydrous salt sintered at 126° and gradually melted. It had $[\alpha]_D^{20^\circ}$ − 231° (c = 1·008 in water). Solubility in water 1 in 19 at 20°. (Found: Cl, 8·4. $C_{22}H_{28}O_4N_2$, HCl requires Cl, 8·4 %.)

n-*Butylquitenine* crystallised from a mixture of benzene and light petroleum (B.P. 60–80°) in anhydrous silky needles, sintering at 137° (corr.), melting at 142° (corr.) and having $[\alpha]_D^{22^\circ}$ − 143° (c = 2·00 in chloroform). (Found: C, 68·9; H, 7·8. $C_{23}H_{30}O_4N_2$ requires C, 69·3; H, 7·6 %.) n-*Butylquitenine monohydrochloride* crystallised from benzene in needles, frothing at 88°. (Found in air-dried salt: loss at 105° in a vacuum, 11·0 %.) The anhydrous salt sintered at 110° and gradually melted. It had $[\alpha]_D^{21^\circ}$ − 227° (c = 0·631 in water) and solubility 1 in 123 of water at 20°. (Found: Cl, 8·2. $C_{23}H_{30}O_4N_2$, HCl requires Cl, 8·2 %.) n-*Butylquitenine dihydrochloride* was not obtained crystalline. The dried substance, which softened at 168° (corr.) and frothed at 178° (corr.), was hygroscopic and had $[\alpha]_D^{18^\circ}$ − 250° (c = 2·045 in water). (Found: Cl, 14·8. $C_{23}H_{30}O_4N_2$, 2HCl requires Cl, 15·0 %.)

iso*Butylquitenine* crystallised from benzene in matted needles, softening at 147° (corr.), melting at 154° (corr.) and having $[\alpha]_D^{20^\circ}$ − 144° (c = 2·02 in chloroform). (Found: C, 68·9: H, 7·8. $C_{23}H_{30}O_4N_2$ requires C, 69·3; H, 7·6 %.) iso*Butylquitenine monohydrochloride* crystallised from benzene in needles, frothing at 93° (corr.). (Found in air-dried salt: loss at 100° in a vacuum, 9·2. $C_{23}H_{30}O_4N_2$, HCl, $2H_2O$ requires H_2O, 7·7 %.) The anhydrous salt sintered at 125°, melted at 248° (corr.) and had $[\alpha]_D^{20^\circ}$ − 223° (c = 1·52 in water). Solubility 1 in 66 of water at 20°. (Found: Cl, 8·1. $C_{23}H_{30}O_4N_2$, HCl requires Cl, 8·2 %.)

n-*Amylquitenine* crystallised from a mixture of acetone and ligroin in needles, softening at 129° (corr.), melting at 135° (corr.) and having $[\alpha]_D^{22^\circ}$ − 135° (c = 2·00 in chloroform). (Found: C, 70·0; H, 8·1. $C_{24}H_{32}O_4N_2$ requires C, 69·9; H, 7·8 %.) n-*Amylquitenine monohydrochloride* crystallised from benzene in minute needles, softening at about 40° and melting at 194° (corr.). (Found in air-dried salt: loss at 100° in a vacuum, 7·6 %. $C_{24}H_{32}O_4N_2$, HCl, $2H_2O$ requires H_2O, 7·4 %.) The anhydrous salt softened at about 115°, resolidified and melted at 195° (corr.). It had $[\alpha]_D^{20^\circ}$ − 217° (c = 0·92 in water) and solubility 1 in 107 of water at 20°. (Found: Cl, 7·9. $C_{24}H_{32}O_4N_2$, HCl requires Cl, 7·9 %.)

iso*Amylquitenine* crystallised from benzene in matted needles, softening at 154° (corr.), melting at 160° (corr.) and having $[\alpha]_D^{21^\circ}$ − 136° (c = 1·989 in chloroform). (Found: C, 69·0; H, 8·0. $C_{24}H_{32}O_4N_2$ requires C, 69·9; H, 7·8 %.) iso*Amylquitenine monohydrochloride* crystallised well from benzene. The air-dried substance frothed at 90°. (Found in air-dried salt: loss at 105° in a vacuum, 19·8 %.) The anhydrous salt softened at 122° (corr.) and gradually decomposed. It had $[\alpha]_D^{17^\circ}$ − 222° (c = 0·804 in water) and solubility 1 in 112 of water at 18°. (Found: Cl, 7·9. $C_{24}H_{32}O_4N_2$, HCl requires Cl, 7·9 %.)

Octylquitenine was obtained in about 10 % yield only, although the proportion of octyl alcohol (methyl-*n*-hexylcarbinol) to quitenine was increased to 50 cc. of alcohol to 5 g. of quitenine, and the esterification was carried out at 110° for 6 hours. The base was not obtained crystalline. *Octylquitenine monohydrochloride* crystallised from benzene in needles, softening at 166° (corr.) and melting at 181° (corr.). (Found in air-dried salt: loss at 105° in a vacuum, 3·6 %. $C_{27}H_{38}O_4N_2$, HCl, H_2O requires H_2O, 3·5 %.) The anhydrous salt had $[\alpha]_D^{21^\circ}$ − 191° ($c = 0{\cdot}244$ in water) and solubility of 1 in 410 of water at 21°. (Found: Cl, 7·2. $C_{27}H_{38}O_4N_2$, HCl requires Cl, 7·2 %.) *Octylquitenine dihydrochloride* was not obtained crystalline. It softened at 136° (corr.) and melted at 164° (corr.). A 1 % aqueous solution could be prepared. (Found: Cl, 13·2. $C_{27}H_{38}O_4N_2$, 2HCl requires Cl, 13·4 %.)

Benzylquitenine was obtained in a yield of about 20 %. After the esterification, water was added and the unchanged benzyl alcohol and benzyl chloride extracted by chloroform. The chloroform solution was washed with water and the washings added to the main acid aqueous solution of the base and unchanged quitenine. The base was liberated by sodium carbonate, extracted by chloroform and recrystallised from a mixture of benzene and light petroleum. The base usually separates as a jelly which changes into anhydrous needles, sintering at 157° (corr.) and melting at 161° (corr.). It had $[\alpha]_D^{24^\circ}$ − 128° ($c = 2{\cdot}002$ in chloroform). (Found: C, 71·7; H, 6·9. $C_{26}H_{28}O_4N_2$ requires C, 72·2; H, 6·5 %.) *Benzylquitenine monohydrochloride* crystallised from alcohol in anhydrous prisms, melting at 233° (corr.), frothing at 238° (corr.), having $[\alpha]_D^{20^\circ}$ − 175° ($c = 0{\cdot}4$ in water) and solubility 1 in 250 of water at 20°. (Found: C, 66·1; H, 6·3; Cl, 7·4. $C_{26}H_{28}O_4N_2$, HCl requires C, 66·6; H, 6·2; Cl, 7·6 %.) *Benzylquitenine dihydrochloride* was not obtained crystalline. It softened at about 160° and melted with frothing at about 200° (corr.). A 10 % aqueous solution could be prepared. (Found: Cl, 13·9. $C_{26}H_{28}O_4N_2$, 2HCl requires Cl, 14·0 %.)

Ethylquitenidine crystallised from acetone in six-sided prisms, sintering at 98° and frothing at 107° (corr.). (Found in air-dried crystals: loss at 120° in a vacuum, 7·4 %. $C_{21}H_{26}O_4N_2$, $2H_2O$ requires H_2O, 8·9 %.) The anhydrous base softened at 131° (corr.), melted at 139° (corr.) and had $[\alpha]_D^{20^\circ}$ + 163° ($c = 2{\cdot}143$ in chloroform). (Found: C, 67·9; H, 7·2. $C_{21}H_{26}O_4N_2$ requires C, 68·1; H, 7·1 %.) *Ethylquitenidine monohydrochloride* crystallised from acetone in anhydrous, apparently cubical, crystals, melting at 242° (corr.), having $[\alpha]_D^{17^\circ}$ + 151° ($c = 1{\cdot}0296$ in water) and solubility 1 in 23 of water at 23°. (Found: Cl, 8·7. $C_{21}H_{26}O_4N_2$, HCl requires Cl, 8·7 %.)

iso*Amylquitenidine monohydrochloride* crystallised from benzene. (Found in air-dried salt: loss at 105° in a vacuum, 2·1 %.) The anhydrous salt melted at 245° (corr.), had $[\alpha]_D^{22^\circ}$ + 132° ($c = 0{\cdot}958$ in water) and solubility in water of 1 in 104 at 22°. (Found: Cl, 7·9. $C_{24}H_{32}O_4N_2$, HCl requires Cl, 7·9 %.)

Ethylcinchotenidine was obtained in 50 % yield under the usual conditions of esterification. It crystallised from acetone in anhydrous rhombic plates, softening at 177° (corr.), melting at 180° (corr.) and having $[\alpha]_D^{20^\circ}$ − 115° ($c = 2{\cdot}081$ in chloroform). (Found: C, 70·3; H, 7·2. $C_{20}H_{24}O_3N_2$ requires

C, 70·5; H, 7·1 %.) *Ethylcinchotenidine monohydrochloride* was not obtained crystalline. It softened at 120°, frothed at 180° (corr.) and had $[\alpha]_D^{20°}$ – 165° (c = 1·3408 in water). It is very soluble in water. (Found: Cl, 9·6. $C_{20}H_{24}O_3N_2$, HCl requires Cl, 9·4 %.)

iso*Amylcinchotenidine monohydrochloride* was not obtained crystalline. It melted at about 152°, had $[\alpha]_D^{21°}$ – 151° (c = 3·788 in water) and solubility 1 in 26 of water at 21°. (Found: Cl, 8·5. $C_{23}H_{30}O_3N_2$, HCl requires Cl, 8·5 %.)

Ethylcinchotenine crystallised from chloroform in anhydrous needles, melting at 213–214° (corr.) and having $[\alpha]_D^{23°}$ + 151° (c = 2·012 in chloroform). (Found: C, 70·2; H, 7·5. $C_{20}H_{24}O_3N_2$ requires C, 70·5; H, 7·1 %.) *Ethylcinchotenine monohydrochloride* crystallised from alcohol in sheaves of plates, frothing at 245° (corr.). (Found in air-dried salt: loss at 105° in a vacuum, 4·6 %. $C_{20}H_{24}O_3N_2$, HCl, H_2O requires H_2O, 4·6 %.) The anhydrous salt frothed at 251° (corr.), had $[\alpha]_D^{17°}$ + 130° (c = 0·982 in water) and solubility 1 in 16 of water at 17°. (Found: C, 63·7; H, 6·8; N, 7·6; Cl, 9·4. $C_{20}H_{24}O_3N_2$, HCl requires C, 63·7; H, 6·7; N, 7·4; Cl, 9·4 %.)

iso*Amylcinchotenine* crystallised from chloroform or benzene in anhydrous needles, melting at 147–149° (corr.). iso*Amylcinchotenine monohydrochloride* was not obtained crystalline. It softened at 129° (corr.), melted at 145° (corr.), had $[\alpha]_D^{20°}$ + 117° (c = 1·3224 in water) and solubility 1 in 10 of water at 20°. (Found: Cl, 8·6. $C_{23}H_{30}O_3N_2$, HCl requires Cl, 8·5 %.)

The solubilities of anhydrous quitenine, quitenidine, cinchotenidine and cinchotenine in water and their optical rotation in 5 % solution in N H_2SO_4 were determined.

	Solubility	Specific rotation
Quitenine	1 in 8300 at 18°	$[\alpha]_D^{25}$ – 298°
Quitenidine	1 in 200 at 23°	$[\alpha]_D^{23}$ + 258°
Cinchotenidine	1 in 277 at 21°	$[\alpha]_D^{25}$ – 207°
Cinchotenine	1 in 233 at 19°	$[\alpha]_D^{26}$ + 209°

The 5-nitrodihydroquinine and 5-aminodihydroquinine were prepared by the methods of Jacobs and Heidelberger [1920].

BIOLOGICAL TESTS.

The biological tests[1] were made in a manner similar to that standardised by Roehl. Canaries were infected with malaria by intraperitoneal inoculation and were subsequently treated daily for 6 days with the compound to be tested. The strain of malaria used was kindly sent to us by Roehl from Elberfeld.

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[1] The biological tests were made at the London School of Hygiene and Tropical Medicine, where one of us (M.) was engaged in investigations on bird malaria on behalf of the Medical Research Council's Chemotherapy Committee, with the aid of grants from the Milner Research Fund and from the Medical Research Council. Our sincere thanks are due to the Director, Sir Andrew Balfour, and to Dr J. G. Thomson for the hospitality of their laboratory.

Thanks to the courtesy of Dr J. G. Thomson we have been able to examine named specimens (sent from Baltimore by Prof. R. Hegner) of the three species of bird-malaria parasite separated by Hartman [1927] and to compare them with the strain obtained from Roehl. The latter corresponded to the type named by Hartman *Plasmodium inconstans*. The doses of the compounds to be tested, dissolved in 0·5 cc. water, were administered to the canaries by means of an oesophageal tube passed into the stomach, and were proportional to the weight of the bird, a bird of 20 g. weight being taken as the standard. The first dose was given 4 hours after the malaria inoculation. As a rule only one bird was treated with each dose of the compound to be tested, and the first tests were made with the largest dose tolerated for 6 consecutive days. Evidence of malaria infection was sought by the examination of stained blood films daily or on alternate days.

In each experiment several birds, usually 20 %, were not treated and were regarded as controls. In the blood of such controls malaria parasites appeared for the first time usually on the 7th or 8th day of the experiment. The virulence of the parasite was somewhat variable, but during 2 years no seasonal variation and no progressive change in virulence was observed. In the birds used as controls during this time the incubation period varied from 3 to 11 days, and averaged 7·3 days; in 49 % the parasites appeared first on the 7th or 8th day, and in 83 % between the 6th and 9th days. Making allowance for this degree of variability in the incubation period, we have adopted an arbitrary standard for estimating the antimalarial action of the compounds tested. A delay in the appearance of parasites as compared with the controls of only 1 or 2 days has been considered negligible, a delay greater than this but not exceeding 7 days has been construed as indicating a "slight" action, and a delay of over 7 days a "moderate" action. The canaries were ordinarily kept under observation for 3 or 4 weeks. If at the end of this time they had shown no evidence of malaria, they were re-inoculated so as to determine whether they had actually been cured by the treatment or were the subjects of a latent infection. If the birds were actually cured, that is malaria-free, the effect of re-inoculation was to produce a normally acute infection, whereas if they were still infected, although not showing parasites in the blood, re-inoculation was without effect. This test presupposes that it is impossible to super-infect with *P. inconstans*, a fact which has been abundantly demonstrated both in the course of this work and by numerous previous investigators. It also assumes that in the course of the 20 days or so intervening between the end of treatment and the day of re-inoculation the drug being tested will have been eliminated from the bodies of the birds. It will be noted that on one or two occasions this assumption was probably incorrect because the first re-inoculation was without effect, whereas re-inoculation at a later date produced a normal acute infection.

The test as outlined above is clearly not a delicate one. It is probably sufficiently trustworthy to distinguish broadly between active and inactive compounds, but for the differentiation of compounds not having widely dissimilar actions, or for the grading of a series of substances with actions of the same order, experiments would have to be made with very much larger numbers of birds.

Table I. *Cinchona alkaloids.*

Drug	Dose in mg. No. of daily doses given	Day on which parasites appeared in blood		Antimalarial action
		Control birds	Treated birds	
Quinine hydrochloride	10·0 × 6	6	—	Cured*
	5·0 × 6	6–8	11–13	Slight
	2·0 × 6	6	14–15	Moderate
	1·0 × 6	7–9	16–17	Moderate
	1·0 × 6	—	12	Slight
	1·0 × 6	6	5	None
	0·5 × 6	5	10	Slight
	0·5 × 6	7–8	9	None
	0·5 × 6	7–9	5	None
Hydroquinine dihydrochloride	5·0 × 6	6–8	—	Cured†
	5·0 × 6	7–8	—	Cured‡
	2·5 × 6	7–8	—	Cured§
	1·0 × 6	7–8	—	Cured‡
	1·0 × 6	5–9	16	Moderate
	1·0 × 6	6	14–15	Moderate
	0·5 × 6	6	11–12	Slight
	0·5 × 6	6	17	Moderate
5-Aminohydroquinine hydrochloride	5·0 × 6	7–8	—	Cured‡
	5·0 × 6	7–8	11–12	Slight
	5·0 × 6	6	12–13	Slight
	1·0 × 6	7–8	12–13	Slight
	0·5 × 6	6–8	7–8	None
Quinidine hydrochloride	10·0 × 6	7–9	22–26	Moderate
	5·0 × 6	7–8	26	— ‖
	5·0 × 6	6–8	13	— ¶
	5·0 × 6	5–9	19–20	Moderate
Dihydroquinidine dihydrochloride	5·0 × 6	6–8	14–16	Moderate
Cinchonine hydrochloride	5·0 × 6	7–8	11–12	Slight
Dihydrocinchonine dihydrochloride	5·0 × 6	6–8	11–13	Slight
Cinchonidine hydrochloride	5·0 × 4 : 2·5 × 2	7–8	16–17	Moderate**
Dihydrocinchonidine hydrochloride	5·0 × 6	7–8	14–16	Moderate

* Re-inoculation on 24th day without effect. Re-inoculation on 46th day produced normal infection, indicating that bird had been cured.

† Re-inoculation on 30th day without effect. Re-inoculation on 40th day produced normal infection, indicating that bird had been cured.

‡ Re-inoculation on 26th day produced normal infection, indicating that bird had been cured.

§ Re-inoculation on 26th day without effect. Re-inoculation on 36th day produced normal infection, indicating that bird had been cured.

‖ Bird died on 26th day without showing evidence of infection.

¶ Bird died on 13th day without showing evidence of infection.

** Dose reduced owing to toxicity.

Cinchona alkaloids (Table I).

Five of these alkaloids have been compared in bird malaria by Giemsa, Weise and Tropp [1926] and the results of the two comparisons may be summarised as follows:

Drug	Giemsa, Weise and Tropp		Present authors	
	Dose (mg.)	Retardation of attack (days)	Dose (mg.)	Retardation of attack (days)
Quinine hydrochloride	2·5–3·0 × 4	12–13	2·0 × 6	8–9
Hydroquinine dihydrochloride	2·5–3·0 × 4	11	2·5 × 6	Cured
,, ,,	—	—	1·0 × 6	7–11 or cured
,, ,,	—	—	0·5 × 6	5–11
5-Aminohydroquinine (base)	2·5–3·0 × 4	11–12	—	—
5-Aminohydroquinine hydrochloride	—	—	5·0 × 6	4–7 or cured
,, ,,	—	—	1·0 × 6	5
Quinidine hydrochloride	1·25–1·5 × 4	12	5·0 × 6	14 or cured
Cinchonine hydrochloride	2·5–3·0 × 4	0–5	5·0 × 6	4–5

Giemsa, Weise and Tropp's results indicate that cinchonine is definitely inferior to any of the other four, which are all of about equal value, whilst our results show that hydroquinine is definitely superior to any of the other four, among which there is little to choose except that there are some signs that quinidine and aminohydroquinine, which both produced cures at a dose of 5 mg., may be better than quinine or cinchonine. From our results cinchonidine also appears to be as good as quinine. The only other investigation which need be mentioned on this point is that of Hegner, Shaw and Manwell [1928], who arrange four of these alkaloids in the following descending order as regards absorption by red blood-corpuscles, which they regard as an indication of efficiency against malaria. The figures are partition coefficients (concentration in corpuscles over concentration in serum) for chicken blood. Quinine, 5·0; quinidine, 4·4; cinchonine, 4·3; cinchonidine, 3·8.

The results of such comparative clinical trials as have been made with the principal cinchona alkaloids are equally confusing.

MacGilchrist [1915–16] arranges them in the following descending order:

$$\text{Hydroquinine} \longrightarrow \begin{array}{l}\text{Cinchonine}\\ \text{Quinine}\\ \text{Quinidine}\end{array} \longrightarrow \text{Cinchonidine}$$

It is remarkable that notwithstanding MacGilchrist's results hydroquinine has not been given a more extended trial in human malaria. The results of our tests on birds show clearly that hydroquinine is much more active than quinine, and suggest that unless its action on human malaria is very different from its action on bird malaria hydroquinine should prove to be of great value in the treatment of the former disease.

Fletcher [1923] says that in doses of 10 grains, twice a day, quinine, quinidine, cinchonine and cinchonidine appear to be of equal value in bringing about the disappearance of malaria parasites, but that in small doses of 5 grains, twice a day, cinchonine did not appear quite so potent as quinine and quinidine, whilst cinchonidine was definitely inferior to the other three.

A comparison of quinine and quinidine made under the auspices of the

Medical Research Council [1925] resulted in the establishment of the practical equivalence of quinine and quinidine, and this view is accepted by the Malaria Commission of the League of Nations [1927], who state that quinine and quinidine are practically of equal value in doses of 1 g. and add that cinchonine is only equally effective in doses of 1·5 g.

Sinton and Bird [1929] on the contrary regard quinine, cinchonine and cinchonidine as of almost equal value in benign tertian malaria and quinidine as inferior to these three.

So far as other alkaloids in Table I are concerned, the results indicate that 5-aminohydroquinine is no better than hydroquinine and that whilst hydrogenation of quinine produces the more active hydroquinine, the hydrogenation of quinidine, cinchonidine and cinchonine does not enhance their antimalarial value. This last observation confirms clinical results recorded by Giemsa and Werner [1914].

It must, however, be pointed out that, so far as bird malaria trials are concerned, the simple technique employed in which a drug is tested on one bird is inadequate. This point is better brought out in Table II, but is also evident in the results recorded in Table I. Thus 5 mg. of quinine hydrochloride gave a retardation of only 5 days in the single bird to which it was given, a less powerful action than that sometimes produced by the smaller doses. A similar variability appears even when, as in Giemsa, Weise and Tropp's experiments [1926], three birds are used. In human malaria the response of patients to treatment with quinine is notoriously variable, a fact usually attributed to variable absorption of the drug from the intestine, and it is reasonable to suppose that birds also would show this variability. Less variable results might be obtained if the drugs could be administered by intramuscular or intravenous inoculation, but this procedure is not practicable for repeated doses of considerable amounts to small and delicate birds. Our experience shows that, using the technique adopted, larger numbers of birds must be used than is at present the custom if trustworthy results are to be obtained.

So far as comparative clinical trials are concerned, there is one point which calls for comment. With the exception of the comparison of quinine and quinidine, made under the auspices of the Medical Research Council, no particular pains seem to have been taken to ensure purity of the alkaloids used. Dawson and Garbade [1930] have recently called attention to this matter in another connection, and if the information they provide as to the extent of the contamination of commercial supplies of individual cinchona alkaloids is accurate, it may account for some of the discrepant results obtained by different observers. Thus, if cinchonidine is, as Dawson and Garbade state, liable to contain up to 10 % of quinine with some hydrocinchonidine, it is conceivable that variation in the amount of these impurities may account for the varying estimates of the antimalarial value of this alkaloid formed by different observers.

Table II. *Esters of quitenine.*

		Day on which parasites appeared in blood		
Drug	Dose in mg. No. of daily doses given	Control birds	Treated birds	Anti-malarial action
Methylquitenine monohydrochloride	10·0 × 6	6–8	11	Slight
	5·0 × 6	7–8	14–15	Slight
	5·0 × 6	6–8	7–8	None
Ethylquitenine monohydrochloride	10·0 × 6	6–8	7–8	None
	5·0 × 6	7–8	9	None
Ethylquitenine dihydrochloride	10·0 × 6	7–9	14–15	Slight
	5·0 × 6	7–8	6	None
n-Propylquitenine monohydrochloride	5·0 × 6	7–8	7–8	None
*iso*Propylquitenine monohydrochloride	10·0 × 6	6–8	12	Slight
	5·0 × 6	7–8	9	None
n-Butylquitenine monohydrochloride	4·1 × 6*	6–8	5	None
n-Butylquitenine dihydrochloride	10·0 × 6	6–8	9–10	Slight
	5·0 × 6	6–8	6	None
	5·0 × 6	6–8	9	Slight
n-Butylquitenine monoacetate	5·0 × 6	7–9	—	Cured†
	5·0 × 6	7–8	14–15	Moderate
	5·0 × 6	7–8	11–12	Slight
	1·0 × 6	7–9	7–8	None
*iso*Butylquitenine dihydrochloride	5·0 × 6	7–8	7–8	None
	5·0 × 6	7–8	12–13	Slight
n-Amylquitenine dihydrochloride	5·0 × 6	7–8	16–17	Moderate
	1·0 × 6	6–11	6	None
*iso*Amylquitenine monohydrochloride	5·0 × 6	7–9	—	Cured†
	5·0 × 6	7–8	10–11	Slight
	5·0 × 6	7–8	9–10	Slight
	1·0 × 6	7–9	10	Slight
*iso*Amylquitenine ethylsulphonate	5·0 × 6	5–9	12–15	Slight
Benzylquitenine monohydrochloride	2·5 × 6*	6	7–8	None
Benzylquitenine dihydrochloride	5·0 × 6	7–8	14–16	Moderate
Benzylquitenine ethylsulphonate	5·0 × 6	6–8	14–15	Moderate
n-Octylquitenine dihydrochloride	2·5 × 6	7–8	7–8	None
Esters of cinchotenidine.				
Ethylcinchotenidine monohydrochloride	5·0 × 6	7–8	12–13	Slight
	1·0 × 6	7–8	5	None
*iso*Amylcinchotenidine monohydrochloride	5·0 × 6	7–8	7–8	None
	5·0 × 6	7–8	14–15	Moderate
Esters of quitenidine.				
Ethylquitenidine monohydrochloride	10·0 × 6	7–9	9	None
*iso*Amylquitenidine dihydrochloride	5·0 × 6	7–8	10	Slight
Esters of cinchotenine.				
Ethylcinchotenine monohydrochloride	10·0 × 6	6	10	Slight
*iso*Amylcinchotenine monohydrochloride	5·0 × 6	7–8	7–8	None
*iso*Amylcinchotenine dihydrochloride	5·0 × 6	7–8	6	None
	5·0 × 6	7–8	7–8	None

* Maximum possible dose owing to low solubility.

† Re-inoculation on 26th day caused normal infection, indicating that bird had been cured.

Quitenine series (Table II).

It has been clearly established already that the free acid itself—quitenine—is inactive both in bird malaria and in human malaria [Stephens *et al.*, 1919], and the object of examining the series of esters of quitenine and the analogous derivatives of the other three cinchona alkaloids dealt with in Table II was to confirm the observation of Giemsa, Weise and Tropp [1926] that the ethyl ester of quitenine is active, and to ascertain whether, by the use of higher alcohols, more active alkylquitenines could be obtained.

The results recorded in Table II make it clear that quitenine does regain activity on esterification and that, on the whole, activity increases as the homologous series is ascended, reaching its maximum at butyl or amyl, at which point cures begin to appear. But, apart from these occasional cures, none of these esters appears to approach hydroquinine or even quinine and quinidine in activity, and the simple quitenine esters do not appear to hold out any hope of a considerable improvement on quinine.

It has been pointed out already by Schnitzer [1926] that esters of quitenine act as local anaesthetics. Dr J. Trevan has kindly tested this point for us. He finds that the esters do act as local anaesthetics, the benzyl ester being markedly active, but that they are too irritating to be of practical value.

We have also examined the esters of the carboxylic acids analogous to quitenine derived from the other three cinchona alkaloids, viz. cinchotenidine from cinchonidine, quitenidine from quinidine and cinchotenine from cinchonine. In these three cases only the ethyl and *iso*amyl esters were used. Here also it is clear, though at most two birds only were used in the trial of each substance, that the esters are active, the best of them being *iso*amylcinchotenidine.

OTHER DRUGS WITH A REPUTATION FOR ANTIMALARIAL ACTION.

Alstonia species. Two of the authors have at present under chemical examination the following species of *Alstonia*:

A. congensis	West Africa.
A. scholaris	India and Philippine Islands.
A. macrophylla	Philippine Islands.
A. constricta	Australia.

The first two contain the alkaloid echitamine [Goodson and Henry, 1925]. The third and fourth contain no echitamine, but are rich in alkaloids, which are still under examination. The total alkaloids of *A. scholaris* showed perhaps a slight action in doses of 2·5 mg., which was the highest dose tolerated, and those of *A. constricta* in doses of 10 mg., but not in doses of 5 mg. Echitamine sulphate was slightly active at a dosage of 5 mg. The total alkaloids of *A. macrophylla* and *A. congensis* were inactive.

Picralima klaineana. The seeds of this plant are regarded by natives in Northern and Western Africa as a cure for malaria. The principal alkaloid present, akuammine [Henry and Sharp, 1927], and the total alkaloids, used as hydrochlorides, proved to have no action in bird malaria.

Nectandra rodioei bark. This material (greenheart bark) is used in British Guiana and elsewhere as a remedy for malaria. The total alkaloids, used as hydrochlorides, had no action in bird malaria.

Indole alkaloids. The alkaloids, echitamine and akuammine, referred to above, are probably both indole derivatives; the former is slightly active. Harmine and harmaline were considered by Gunn and Marshall [1920] to be active in human malaria. Both were tried in this series of experiments and found to be inactive in bird malaria in doses of 2·5 or 5·0 mg.

iso*Quinoline derivatives.*

Among the alkaloids, which have at one time or another been used for the treatment of malaria, is narcotine, which belongs to the *iso*quinoline group. It seemed worth while, therefore, to try a series of *iso*quinoline alkaloids in bird malaria and the following were used.

Papaverine hydrochloride
Tetrahydropapaverine hydrochloride
Pavine hydrochloride
N-Ethyltetrahydropapaverine hydrochloride
N-Methylpavine hydrochloride
N-Methylpavine methochloride
Laudanosine hydrochloride
Aminolaudanosine hydrochloride
Berberine phosphate
Anhydromethylcanadine

None of these had any traceable preventive or curative action. It has been suggested by Waldorp [1926] that berberine is a useful drug for provoking latent malaria into activity, but no such effect was observed in these experiments.

Miscellaneous drugs.

Hegner, Shaw and Manwell [1928] have already tried a large number of miscellaneous drugs in bird malaria without finding anything outside the cinchona alkaloid series which seemed promising, with the possible exception of one organic mercury compound.

In spite of these discouraging results, it was considered worth while to try a number of compounds, which we had available. These were: pseudaconine, conessine, daphnandrine, acriflavine, proflavine, sparteine.

There was no reason to expect protozooicidal activity in the case of pseudaconine and daphnandrine, but conessine is now known to be very toxic to *Entamoeba histolytica*, and sparteine has long been supposed to resemble the cinchona alkaloids in possessing a quinuclidine structure. None of these six substances showed any activity in bird malaria.

SUMMARY.

1. Out of an extensive series of alkaloids tested on canaries infected with *Plasmodium inconstans* only the alkaloids belonging to, or derived from, the cinchona series showed marked remedial action.

2. Of the natural cinchona alkaloids the most active was hydroquinine followed by quinidine, quinine, cinchonidine and cinchonine in descending order, though there is little to choose among the last four. Hydrogenation of quinidine, cinchonidine and cinchonine does not appear to lead to increased activity, as it does in the case of quinine.

3. Activity is restored to quitenine and the analogous carboxylic acids derived from quinidine, cinchonidine and cinchonine, by esterification, and there are clear indications of a rise in activity as the molecular weight of the alcohol used for esterification is increased, which reaches a maximum at butyl- or amyl-quitenine.

4. Among other alkaloidal drugs having some reputation as remedies for malaria, activity was observed only in two *Alstonia* species, viz. *A. scholaris* and *A. constricta*, and there it was slight. The former species no doubt owes its activity to echitamine, which exhibited slight action in doses of 5 mg.

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XCIX. THE INACTIVATION OF PANCREATIC LIPASE BY HEAT.

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Very little is known about the heat-inactivation of pancreatic lipase, and the work that has been done is rather qualitative than quantitative. Terroine [1910] observed that pancreatic secretion was very sensitive to heat and rapidly lost its lipolytic activity at 45°. At 65° the lipolytic activity was completely lost in 10 minutes. According to the same author bile salts accelerated this process. Terroine also investigated the effect of activating the trypsin in the secretion with enterokinase, and observed that the lipase was then more rapidly destroyed. The addition of coagulated egg-albumin decreased the action of the trypsin-kinase on the lipase. Mellanby and Woolley [1914] have observed that pancreatic secretion, of alkalinity 0·12 N sodium carbonate, lost its lipolytic activity at the rate of 10 % per hour at 40°. At 50° the rate was 50 % per hour and at 60° the whole of the lipolytic activity was lost within 5 minutes. The material was found to lose its activity at a slightly slower rate when the solution was neutral. When the solution was made 0·02 N with respect to HCl it was found to be destroyed very rapidly. These authors also observed that the rate of inactivation was increased when enterokinase was added, and that serum-albumin and egg-albumin protected the lipase from destruction. Lombroso [1914] observed that in the presence of such substances as glycerol, glucose, sucrose and maltose (compounds which are heavily hydrated in solution) pancreatic lipase was much more stable to heat. Willstätter and Waldschmidt-Leitz [1923] employed glycerol as the most efficient stabiliser of pancreatic lipase in their experiments on the purification of this enzyme. The work described in this paper deals with a quantitative investigation of the heat-inactivation of pancreatic lipase.

THE ESTIMATION OF PANCREATIC LIPASE.

It is necessary in the first place to have some reliable method for the estimation of the lipase. The materials employed in the estimation were prepared as follows.

(*a*) *Glycerol extract of lipase.* Dried gland powder was prepared by drying pig's pancreas with acetone and ether according to the procedure of Willstätter and Waldschmidt-Leitz [1923]. 6 g. of this dried gland powder were added to 100 cc. glycerol-water mixture (containing 80 % glycerol), the mixture then being kept with occasional shaking at 30° for 4 hours. After centrifuging, the supernatant liquid was clarified by filtration through two layers of filter paper. The clear liquid thus obtained is referred to as a "glycerol extract of lipase."

(*b*) *Neutral olive oil.* The method employed was a modification of that due to Corran and Lewis [1928]. Commercial olive oil (Boots's finest Lucca olive oil) was shaken with an equal volume of 1 % sodium hydroxide solution in a separating funnel at about 75°. After standing, the aqueous layer was separated, and the emulsion of oil remaining washed with hot distilled water until all soap was removed. The oil was then dried by heating on a water-bath at 2 mm. pressure. The same stock commercial olive oil was employed throughout, and the saponification number 192·5 was found to be unaltered in different preparations[1].

(*c*) *Egg-albumin solution.* 2 g. of B.D.H. egg-albumin powder were dissolved in excess ammonia, the ammonia then being evaporated by impinging a stream of warm air (30°) on the surface of the liquid. The solution was left overnight in a vacuum desiccator containing sulphuric acid and then made up to 100 cc. It is referred to as 2 % albumin solution. The p_H of such a solution was found to be approximately 7·6.

(*d*) *Calcium chloride solution.* A solution of calcium chloride was made from pure fused calcium chloride free from calcium hydroxide. The solution was adjusted to contain 1 % calcium chloride by estimating the Cl ion concentration and diluting the solution accordingly.

(*e*) *Sodium chloride solution.* A 0·1 N solution of sodium chloride was prepared from B.D.H. sodium chloride which had been purified by precipitation from a saturated solution with HCl gas and dried at 105° for 3 hours.

(*f*) *Buffer solution.* The buffer described by Willstätter, Waldschmidt-Leitz and Memmen [1923] was made up by mixing 66 parts of N ammonia and 134 parts of N ammonium chloride. The p_H of this solution was measured with the glass electrode and found to be 8·9 at 30°.

Willstätter, Waldschmidt-Leitz and Memmen [1923] have observed that calcium chloride and egg-albumin are excellent activators of pancreatic lipase in an alkaline medium and employ these substances in their method of estimation.

The writer has verified the excellent activations of pancreatic lipase obtained with calcium chloride and egg-albumin, and numerous experiments on the combined action of the two activators have been carried out. The combined effect of the two was found to be slightly greater than the sum of the two separate effects, again confirming the results of the above authors. The experiments were carried out as described by these investigators. The enzyme material was diluted with sufficient water to keep the total volume of the water solution 13 cc. 2·5 g. olive oil, 2 cc. buffer solution and the calcium chloride solution were added and the mixture was gently shaken. Finally, the solution of egg-albumin was added and the mixture shaken vigorously by hand for 3 minutes and then placed in a thermostat at 30° for 57 minutes. The contents of the reaction vessel were washed into an Erlenmeyer flask with 96 % alcohol so that the volume of the alcoholic liquid was 125 cc. 20 cc. ether were added and the acid titrated with 0·1 *N* alcoholic NaOH in the presence of thymolphthalein. Control experiments in which the buffer and protein of the enzyme material were titrated were also carried out to determine the degree of hydrolysis of the olive oil. A few of the results are given in the table below.

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[1] The writer is indebted to Mr Priestman, of the Department of Industrial Chemistry, the University of Liverpool, for these determinations.

Table I. *Temp. 30°. Enzyme extract 0·4 cc. Total volume of water solution 13 cc.*

mg. $CaCl_2$	mg. albumin	Hydrolysis in cc. 0·1 *N* NaOH	mg. $CaCl_2$	mg. albumin	Hydrolysis in cc. 0·1 *N* NaOH
0	0	6·5	0	10	8·2
10	0	10·95	15	10	14·4
10	10	12·8	20	10	16·4
10	15	13·6	0	20	9·5
10	20	14·6	20	20	17·1
10	40	15·4			

In the presence of a high concentration of calcium chloride (greater than 15 mg.) a thick creamy mass, which contained all the oil, separated out when the shaking was stopped, and the reproducibility of the experiments was not so good as that obtained with smaller concentrations of calcium chloride, in which case good emulsification of the oil resulted. In general, the writer considered 10 mg. calcium chloride and 20 mg. egg-albumin in a total volume of 13 cc. water solution to be a good combination of activators to employ to swamp the effect of the substances accompanying the enzyme.

An investigation of the heat-inactivation of pancreatic lipase would necessarily involve the use of glycerol extracts. This might mean a considerable variation in the concentration of the glycerol in the determination mixture during such an investigation (*e.g.* only 0·2–0·4 cc. of a fresh glycerol (80 % glycerol) extract are sufficient to give a suitable hydrolysis, whereas after heating at 55° for 30 minutes 1·0–1·5 cc. are required to give the same hydrolysis). Willstätter, Waldschmidt-Leitz and Memmen [1923] observed that glycerol accelerated the action of pancreatic lipase under the conditions of the experiments, *i.e.* an initial p_H of 8·9. They, however, only worked with 0·2–0·4 cc. of an 87 % glycerol extract, and the control of the glycerol concentration was unnecessary. The writer has investigated the effect of glycerol on the action of pancreatic lipase in the absence of other activators and also in the presence of 10 mg. calcium chloride and 20 mg. egg-albumin. The glycerol employed was B.D.H. (A.R.) glycerol. The results are given in Tables II and III.

Table II. *The effect of glycerol in the absence of other activators. Temp. 30°. Enzyme extract 0·4 cc. Initial* $p_H = 8·9$. *Total volume water solution = 13 cc.*

cc. glycerol	Hydrolysis in cc. 0·1 *N* NaOH	cc. glycerol	Hydrolysis in cc. 0·1 *N* NaOH
0	6·3	3·0	15·5
1·0	9·4	4·0	17·8
2·0	12·1	5·0	19·2

Table III. *The effect of glycerol in the presence of 10 mg. calcium chloride and 20 mg. egg-albumin. Temp. 30°. Enzyme extract 0·2 cc. Initial* $p_H = 8·9$. *Total volume of water solution = 13 cc.*

cc. glycerol	Hydrolysis in cc. 0·1 *N* NaOH	cc. glycerol	Hydrolysis in cc. 0·1 *N* NaOH
0	15·3	3·0	16·5
1·0	16·0	4·0	16·6
2·0	16·3		

(The extracts employed in Tables II and III are similar but not identical.) It will be seen that the action of glycerol is not so marked in the presence of calcium chloride and albumin as when these activators are absent. Similar results were obtained when different enzyme concentrations were employed. Although the effect in the presence of calcium chloride and albumin is small, it was considered necessary to keep the concentration of the glycerol constant in a mixture for the determination of lipase. The quantity chosen was 2 cc. of pure glycerol. This quantity allows for the use of 4 cc. of a 50 % glycerol extract without bringing in any error due to the acceleration produced by uncompensated glycerol concentration.

Another factor which must be considered is the method of regulating the p_H when carrying out the heat-inactivation of lipase at different p_H values. By employing NaOH and HCl a wide range of p_H values may be obtained. The p_H in the method of estimation of the lipase proposed to be employed is essentially a changing one, the 2 cc. of buffer employed to give an initial p_H of 8·9 being insufficient to keep the p_H constant. It is therefore to be expected that the estimation will not give true values for the amount of lipase if the enzyme material contains large varying amounts of NaOH or HCl. (The protein in the extracts, especially in the water extract, makes it necessary to employ a considerable quantity of acid or alkali to alter the p_H appreciably, *e.g.* 1 cc. water extract of p_H 5·93 requires 2·1 cc. 0·1 *N* NaOH to bring the p_H to 9·5.) If the NaOH or HCl were neutralised prior to the actual process of estimation a certain quantity of NaCl would be present. It is therefore necessary to carry out experiments to see if NaCl has any effect on the action of lipase upon olive oil in the presence of 10 mg. calcium chloride and 20 mg. egg-albumin. The results are given in Table IV.

The effect is sufficient to necessitate the use of a constant concentration of NaCl in the estimation mixture. The quantity chosen was 1 cc. of 0·1 *N* sodium chloride in a total volume of 13 cc. water solution.

Table IV. *Initial* p_H = *8·9. Enzyme extract 0·3 cc. Temp. = 30°. Total volume water solution = 13 cc.*

cc. 0·1 N NaCl	Hydrolysis in cc. 0·1 N NaOH	cc. 0·1 N NaCl	Hydrolysis in cc. 0·1 N NaOH
0	17·7	0·75	18·3
0·25	17·85	1·0	18·4
0·5	18·15		

The conditions for the method of determination now being fixed it only remains to find a relation between the enzyme quantity and the degree of saponification.

The reactions were carried out in Erlenmeyer flasks of 50 cc. capacity fitted with tight-fitting rubber stoppers. Sufficient water and pure glycerol were added to the enzyme material to make the volume of the water 6 cc., and that of the glycerol 2 cc. 1 cc. 0·1 N NaCl, 2·5 g. olive oil, 2 cc. buffer and 1 cc. 1 % (10 mg.) calcium chloride were added and the mixture was shaken for a suitably short time (5 secs.). 1 cc. of 2 % (20 mg.) egg-albumin solution was then run in, and the mixture vigorously shaken by hand for 3 minutes. The reaction flasks were then placed in a thermostat at 30° for 57 minutes and tested. The contents were washed into Erlenmeyer flasks of 300 cc. capacity with 96 % alcohol so that the volume of the alcoholic liquid was 125 cc. 20 cc. ether were added and the acid was titrated with 0·1 N alcoholic NaOH in the presence of thymolphthalein as indicator. The degree of hydrolysis obtained with different quantities of enzyme material, under the above conditions, was investigated and a graph plotted showing the relation between the degree of saponification and the quantity of enzyme employed.

The results, given in Table V, are means of several readings.

Table V.

Enzyme expressed in cc. glycerol extract	Hydrolysis in cc. 0·1 N NaOH	Enzyme expressed in cc. glycerol extract	Hydrolysis in cc. 0·1 N NaOH
0·05	9·70	0·25	18·25
0·10	13·80	0·30	19·1
0·15	15·25	0·40	20·75
0·20	17·1	0·50	21·60

All enzyme extracts are not of the same activity, hence one does not obtain the same hydrolysis by using the same quantity of different extracts. For purposes of standardisation Willstätter and Kuhn [1922] have suggested an arbitrarily chosen measure which is termed an enzyme unit. The writer in adopting this method defines a lipase unit as the quantity of enzyme which produces a saponification of 2·5 g. of olive oil, equivalent to 20 cc. 0·1 N sodium hydroxide, in 1 hour at 30° under the conditions specified above. The adoption of this unit does not mean, however, that a given enzyme extract containing say 0·5 unit will produce just half the amount of saponification in 1 hour at 30°. The actual degree of saponification produced by 0·5 (or any other submultiple or multiple) of a unit has to be determined by experiment. This is effected in principle by starting with a given quantity of extract, and finding by trial what volume of this extract contains just one lipase unit as defined above. Aliquot portions of this amount of extract are then examined for their saponifying powers, and a graph constructed connecting number of units with cc. of alkali required (Fig. 1). Using extracts from different glands, and proceeding in the above manner, a series of identical curves was obtained, the reproducibility being satisfactory.

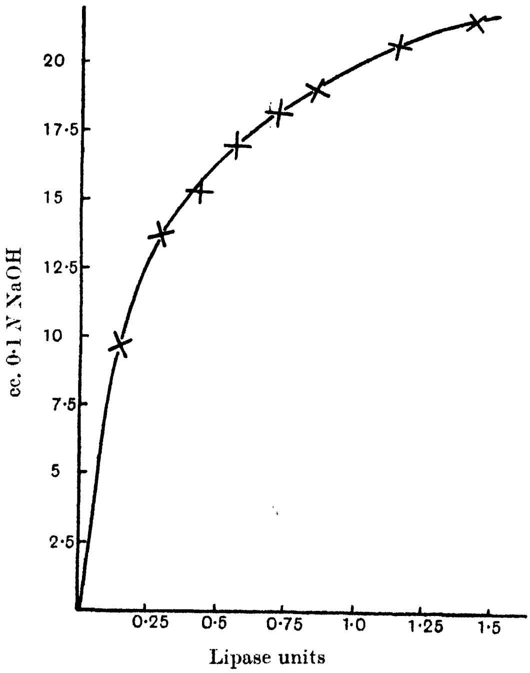

Fig. 1.

It may be mentioned that the unit as defined above is necessarily different from that of Willstätter, Waldschmidt-Leitz and Memmen, since the conditions of the experiment are different; the shape of the curve is not the same as that obtained by these authors.

THE HEAT-INACTIVATION OF PURIFIED LIPASE.

(a) *Preparation of purified lipase.*

In order to obtain a purified lipase the writer has employed a modification of the method of Willstätter and Waldschmidt-Leitz [1923], who obtained lipase free from trypsin and amylase by carrying out two successive adsorptions on the aluminium hydroxide B of Willstätter and Kraut [1923]. They observed that a mixture containing 57 parts by volume of 1 % diammonium phosphate, 3 parts of *N* ammonia and 40 parts of 87 % glycerol was most suitable for eluting the enzyme from the alumina-lipase adsorption complex. It was found impossible to expel the inorganic salts by dialysis against distilled water, as the lipase was completely destroyed during the process.

The method employed by the writer was as follows. Two successive adsorptions were carried out in exactly the same way as described by Willstätter and Waldschmidt-Leitz. After the second adsorption, however, the lipase was eluted from the alumina-enzyme adsorption complex with a mixture of 40 parts of glycerol, 3 of *N* ammonia and 57 of water. The ammonia was then evaporated *in vacuo* at 25°, and the solution placed in a desiccator over sulphuric acid, the p_H being thus brought to 7·0. This solution adjusted to contain 50 % glycerol was employed as a purified lipase. It was found to be free from trypsin-kinase. The yield was not as good as that obtained with the mixture containing ammonium phosphate, about 20 % of the lipase of the original glycerol extract being obtained. The absence of phosphate, however, is desirable in the present instance.

(*b*) *Apparatus employed.*

The apparatus employed is shown in Fig. 2.

The reaction vessel *A* is a silica vessel of about 70 cc. capacity, closed by a rubber bung *B*. *C* is a tube through which samples are withdrawn from time to time. Air enters the vessel *A* by means of the side arm *F*, which leads from vessel *G*, which contains the same solution as that under investigation. Air entering this vessel does so by way of a vertical tube *H* which dips under the surface of the liquid. Both vessels are kept well immersed in the water of the thermostat which is regulated to ± 0·05°. This apparatus prevents any change of concentration due to evaporation during an experiment.

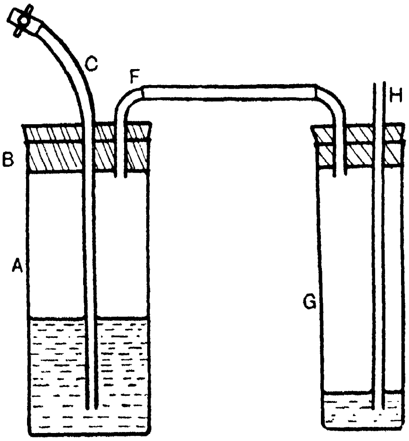

Fig. 2.

A definite quantity of lipase extract is taken and the p_H regulated by the addition of NaOH or HCl of known strength. This solution is placed in vessel *A* which is already at the temperature of the thermostat. Portions of the extract are withdrawn at various intervals of time and run into test-tubes standing in crushed ice. The first withdrawal is made 2–3 minutes after placing the extract in the thermostat. The lipase content of the various portions is estimated according to the method already described.

(*c*) *Course of the reaction.*

The applicability of the unimolecular expression to the heat-inactivation of this preparation of pancreatic lipase was tested by numerous experiments. The general behaviour is illustrated by Table VI.

The values of k_{uni} were calculated from the expression

$$k_{\text{uni}} = \frac{1}{t} \log_e \frac{a}{a - x},$$

where t = time in minutes, a = lipase units per cc. at zero time and $a - x$ = lipase units per cc. at time t.

Table VI.

Time in mins.	Lipase units/cc.	$k_{\text{uni}} \times 10^2$
Temp. 50°; 50 % glycerol; $p_H = 6{\cdot}00$		
0	0·365	
5	0·250	7·57
10	0·183	6·90
15	0·130	6·90
Temp. 40°; 50 % glycerol; $p_H = 8{\cdot}00$		
0	0·42	
30	0·29	1·23
60	0·225	0·90
90	0·15	1·14

It was observed that on the whole the constant decreased slightly in many of the experiments.

(*d*) *The effect of* p_H *upon the heat-inactivation.*

The effect of the p_H of the medium[1] is shown in Table VII and Fig. 3. The value for k_{uni} at a particular p_H is that obtained by taking the mean of several readings. It will be seen that the enzyme is most stable in the region of p_H 6·0.

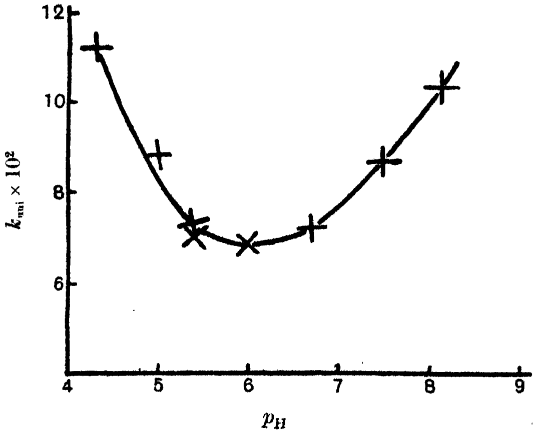

Fig. 3.

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[1] The p_H measurements throughout this work were made by means of the glass electrode by the method described by Millet [1928].

Table VII.

Temp. 50°; 50 % glycerol

p_H	$k_{uni} \times 10^2$	p_H	$k_{uni} \times 10^2$
8·14	10·3	5·41	7·00
7·48	8·7	5·40	7·24
6·75	7·21	4·97	8·80
6·03	6·83	4·23	11·14

(e) *The critical increment of the process.*

In order to determine the critical increment for the heat-inactivation of purified pancreatic lipase, measurements were carried out at three different p_H values: (1) at the region of optimum stability, p_H 6·00; (2) at p_H 8·01; and (3) at p_H 5·00.

The results are given in Tables VIII, IX, X. The critical increment was calculated by substituting the values of k_{uni} into the integrated form of the equation

$$\frac{d \ln k}{dT} = \frac{E}{RT^2}.$$

Table VIII.

50 % glycerol; p_H = 6·0

Time in mins.	Lipase units/cc.		$k_{uni} \times 10^3$
	Temp. 40°		
0	0·475		6·83
60	0·315		6·80
120	0·21		6·59
180	0·145		
		Mean =	6·74
	Duplicate exp.	Mean =	6·84
		Average =	6·80
	Temp. 50°		
0	0·365		75·7
5	0·250		69·0
10	0·183		69·0
15	0·130		
		Mean =	71·2
	Duplicate exp.	Mean =	70·7
		Average =	70·8

From these values of k_{uni} we arrive at the value of E = 46,000 calories at p_H 6·00.

Table IX.

Glycerol content of solution 50 %. p_H = 8·01.
Temp. 40°. Mean value of $k_{uni} = 1{\cdot}14 \times 10^{-2}$.
Temp. 50°. Mean value of $k_{uni} = 10{\cdot}1 \times 10^{-2}$.

From the above values of k_{uni} at 40° and 50° the value for the critical increment is found to be 44,000 calories at p_H 8·01.

Table X.

Glycerol content of solution 50 %. $p_H = 5{\cdot}1$.
Temp. 40°. Mean value of $k_{uni} = 7{\cdot}95 \times 10^{-3}$.
Temp. 50°. Mean value of $k_{uni} = 7{\cdot}80 \times 10^{-2}$.

The critical increment calculated from these values is 45,800 calories at p_H 5·1.

Table XI. *The critical increment in 25 °/₀ glycerol.*

Glycerol content of solution 25 %. $p_H = 6{\cdot}00$.
Temp. 40°. Mean value of $k_{uni} = 7{\cdot}90 \times 10^{-3}$.
Temp. 50°. Mean value of $k_{uni} = 8{\cdot}60 \times 10^{-2}$.

The critical increment calculated from these data is 47,800 calories.

The results obtained with purified lipase are summarised in Table XII.

Table XII. *Purified lipase.*

Glycerol concentration %	p_H	E in calories
50	6·00	46,000
50	8·01	44,000
50	5·1	45,800
25	6·00	47,800

Before discussing the results obtained above a very brief statement of the experiments carried out with unpurified lipase will be given.

THE HEAT-INACTIVATION OF UNPURIFIED LIPASE.

Experiments were carried out with unpurified extracts of lipase in order to compare the results with those obtained with the pure enzyme.

These extracts were prepared by extracting 6 g. of the dried gland powder with 100 cc. of water or water-glycerol mixture for 2–4 hours at 30°. (Water gives a good extraction in 2 hours, while 80 % glycerol requires a longer time.) The mixture was then centrifuged and the supernatant liquid clarified by filtration.

In 80 % glycerol it was found that the course of the reaction follows the unimolecular expression quite satisfactorily except at p_H values on the acid side of the p_H of optimum stability ($p_H = 6{\cdot}0$) (Table XIII). In attaining these p_H values by the addition of HCl a precipitate was formed. (No precipitate was formed on adding NaOH.) This precipitate would carry along with it some of the lipase, and presumably this portion of the lipase is less liable to inactivation. At $p_H = 5{\cdot}0$ the velocity recorded is the initial value (since in this case the k falls with time).

Table XIII.

Temp. 50°; 80 % glycerol.

p_H	$k_{uni} \times 10^3$	p_H	$k_{uni} \times 10^3$
5·00	26·05	8·20	34·3
5·99	7·18	9·20	48·8
7·20	17·26	10·23	162·8

The results show that the enzyme is most stable in the region of p_H 6·0, as was the case with the purified enzyme in 50 % glycerol.

In the case of the water extract the unimolecular constant was found to decrease markedly with time at all p_H values. Thus at 40° and p_H 5·93 the "constant" decreased 50 % in 90 minutes. This decrease in the constant is more marked at 50°, while at 30° the decrease in 60 minutes only amounts to 15 %. The extract was most stable in the region of p_H 6·0.

The critical increment for the process was determined by measuring k_{uni} at different temperatures both in the presence and in the absence of glycerol. In the case of water extracts the initial values of k_{uni} were used to calculate the critical increment.

The results are summarised in Table XIV.

Table XIV. *Unpurified lipase.*

Glycerol conc. %	p_H	Temp. interval in ° C.	E in calories
80	5·99	45–50	101,000
80	5·99	50–55	103,000
80	9·30	45–50	92,000
50	5·98	45–50	57,500
0	5·93	30–40	35,000

DISCUSSION OF RESULTS.

In agreement with the results obtained for other enzymes the p_H of optimum stability of purified pancreatic lipase does not coincide with its "optimum p_H," *i.e.* p_H of optimum activity upon a substrate. The optimum thermal stability is in the region of p_H 6·0, whereas the "optimum p_H" is 8·0. The same behaviour is exhibited by the unpurified material.

In general it may be said that the behaviour of the purified extract is regular and reproducible. On the other hand, in the absence of glycerol the behaviour of the unpurified extract would depend upon the amount of trypsin-kinase present. The trypsin-kinase content of the unpurified material was determined according to the method of Willstätter *et al.* [1926], but without activation with enterokinase. It was found that a water extract of the "dried gland powder" contained 1·97 trypsin units per cc. and the 80 % glycerol extract 0·48 trypsin units per cc.[1]

Willstätter and Persiel [1924] have shown that glycerol inhibits the action of trypsin-kinase, hence this proteolytic enzyme would not be expected to act in 80 % glycerol. The observations of Terroine [1910] and Mellanby and Woolley [1914] indicate that trypsin activated with enterokinase (*i.e.* trypsin-kinase) destroys lipase. During the process of heat-inactivation of an impure water extract the writer has observed that a small amount of acid is formed, whereas in the 80 % glycerol extract no measurable amount results. In an 80 % glycerol extract, as would therefore be expected, the process of heat-inactivation, even of the unpurified material, is unimolecular. In a water extract, however, there are two processes going on: (1) the destruction of the lipase by heat; (2) the destruction of the lipase by trypsin-kinase. A unimolecular velocity constant for heat-inactivation is therefore not to be expected in this case.

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[1] The writer is indebted to Mr Pace, of the Department of Physical Chemistry, University of Liverpool, for these determinations.

It is also suggested that the change in the critical increment for the heat-inactivation of unpurified lipase with change in glycerol content is due to the action of trypsin-kinase, this enzyme destroying the protein carrier and thereby inactivating the active grouping of the lipase. In 80 % glycerol the trypsin-kinase effect is minimal and the observed critical increment of the heat-inactivation of unpurified lipase is very large—of the order 90,000–100,000 calories. In water the trypsin-kinase effect ought to be maximal. The critical increment of hydrolysis of caseinogen by trypsin-kinase is of the order 14,000 calories [Moelwyn-Hughes, Pace and Lewis, 1930]. This low value is probably the reason why the apparent critical increment of heat-inactivation of unpurified lipase in water is as low as 35,000 calories.

The most interesting observation is the relatively low value for the heat-inactivation of purified lipase, namely, 46,000 calories. As this value is obtained in 50 % and 25 % glycerol it seems reasonable to assume that much the same value holds in water. This value should be compared with 90,000–100,000 calories obtained for the unpurified lipase, under conditions in which the trypsin-kinase is ineffective (*i.e.* in 80 % glycerol). The effect of purification is therefore to reduce the critical increment to approximately one-half. This naturally accounts for the fact that the purified material is much less stable than the unpurified. At the same time, one is at a loss to explain precisely the manner in which this reduction in critical increment has been brought about by the act of purification. The purification consists mainly in the removal of protein, and this change in the accompanying substances in some way causes a lowering in the critical increment[1]. It is interesting to note that the critical increment of purified lipase is of the same order as that obtained by Pace [1930] for the heat-inactivation of trypsin itself, free from enterokinase and the pre-stage of this activator, the value obtained being 40,000 calories.

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[1] The unpurified lipase has protective impurities adsorbed or otherwise combined with it. The extent of such adsorption will be a function of temperature, adsorption in general diminishing as temperature rises. Hence at the higher temperature the unpurified lipase is now partly purified by removal of impurity and hence is thermally inactivated at a greater rate than would have been found had the extent of adsorption remained independent of temperature. It follows that a correspondingly high value (90,000–100,000 cals.) is obtained for the apparent critical increment. If this point of view be the correct one, it may be expressed in a quantitative form. Thus if Q is the (positive) heat evolved in the adsorption of the impurities on one "molar" unit of enzyme, E_0 the true critical increment of heat-inactivation of purified enzyme and E_{obs} the observed value for the unpurified enzyme, then we would expect

$$E_{\text{obs}} = E_0 + Q.$$

Setting $E_{\text{obs}} = 95{,}000$, $E_0 = 45{,}000$ cals., it follows that $Q = 50{,}000$ cals. This is a very high value for adsorption but it has to be remembered that the "molecular unit" of an enzyme is exceedingly large and the number of molecules of adsorbed material per enzyme unit correspondingly great.

SUMMARY.

1. A modification of the method of Willstätter, Waldschmidt-Leitz and Memmen for the estimation of pancreatic lipase has been employed in investigating the heat-inactivation of this enzyme. The relation between the concentration of the enzyme and the degree of hydrolysis has been obtained under the new conditions, and a lipase unit, necessarily different from that of the above authors, has been defined and employed in the later work described.

2. A purified lipase has been prepared by two adsorptions on aluminium hydroxide. In order to obtain a preparation free from salts an elution mixture of glycerol, water and ammonia is employed in eluting the enzyme after the second adsorption.

3. Experiments have been carried out on the heat-inactivation of this purified lipase. The course of the heat-inactivation process was found to be unimolecular.

4. The effect of p_H upon the heat-inactivation of lipase prepared in the above manner has been investigated. It has been found that the optimum stability of the lipase is about p_H 6·0.

5. The critical increment for the heat-inactivation process in 50 % glycerol has been determined at three p_H values: 6·0, 8·01 and 5·0. It was found that the critical increment was sensibly the same at all three points, and was of the order of 46,000 calories.

6. The critical increment was likewise determined in 25 % glycerol and p_H 6·0 and was found to be 47,800 calories. It is concluded therefore that in the absence of glycerol the critical increment would be of this order.

7. Experiments were also carried out on the heat-inactivation of extracts of unpurified lipase. The course of the reaction in 80 % glycerol was found to be unimolecular, whereas in the water extract of the "dried gland powder" the unimolecular constant was found to decrease with time. This decrease is explained by the action of the trypsin-kinase in the water extract.

8. The effect of p_H upon an 80 % glycerol extract and water extract of unpurified lipase has been investigated. It was found that the enzyme is most stable at about p_H 6·0.

9. At p_H 6·0 and in 80 % glycerol the critical increment for the process in unpurified extracts is shown to be of the order of 100,000 calories, whereas in 50 % glycerol the value is 57,500 calories, and in water 35,000 calories. This change in the critical increment with change in the concentration of glycerol is ascribed to the action of the trypsin-kinase. The critical increment for the heat-inactivation process of an unpurified extract containing 80 % glycerol at p_H 9·3 is of the order 92,000 calories, which is sensibly the same as 100,000 calories.

10. The fall from 95,000 calories for unpurified lipase to 45,000 calories for purified lipase is in agreement with the increased instability of the purified material.

The work described in this paper was carried out under the direction of Prof. W. C. M. Lewis, to whom the writer is indebted for help and advice. The writer also wishes to thank the Trustees of the H. B. Webb Fund, of the University of South Africa, for the award of a Research Scholarship, and is indebted to Imperial Chemical Industries, Ltd., for a grant to the Department of Physical Chemistry of the University of Liverpool.

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C. CARBOHYDRATE METABOLISM OF NORMAL AND TUMOUR TISSUE.

PART I. A METHOD FOR THE MEASUREMENT OF THE RESPIRATORY QUOTIENT.

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METABOLIC measurements upon isolated tissues *in vitro* have attained a high degree of accuracy in so far as the consumption of oxygen, or the measurement of glycolysis, alone is concerned. However, when the measurement of CO_2 actually formed in the tissues is considered, the existing methods are found to be generally unsatisfactory. Since the whole significance of the respiratory quotient is dependent upon very slight variations in its numerical value, it is of the utmost importance that the accuracy of the determination of CO_2 should be as nearly as possible equal to that of the oxygen measurement. The methods in common use are as follows.

(1) The oxygen consumption of a piece of tissue is measured manometrically, the CO_2 being absorbed by KOH. Comparison of the readings with those obtained with another piece of tissue in a second manometer vessel without KOH allows the CO_2 to be calculated [Büchner and Grafe, 1924]. A differential arrangement may also be used, as in Thunberg's respirometer [Fenn, 1927].

(2) In order to overcome the difficulties due to insufficient buffering and at the same time to use a physiological concentration of bicarbonate in the medium, Warburg introduced his "improved method" [Warburg, 1926]. This method is based on the different solubilities of oxygen and CO_2, and the respiration is calculated from readings made with two vessels with similar pieces of tissue but with different volumes of fluid in each. It is of great value for the measurement of aerobic glycolysis but cannot be used for the true respiratory quotient, since the figure for CO_2 obtained includes the "extra CO_2" liberated from the bicarbonate by the production of acid, in addition to the respiratory CO_2. Referring to the estimation of true respiratory quotient in bicarbonate-containing solutions, Warburg [1926] states: "On account of the high physiological bicarbonate concentration it is difficult to estimate the increase of bound CO_2 with sufficient accuracy, and it is questionable whether this difficulty can be overcome by a differential procedure."

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(3) Recently Crabtree [1929] has described a method in which the "improved method" of Warburg is combined with estimation of chemically bound CO_2 by acidification before and after the experiment. The objection quoted above greatly limits the accuracy of this method, since the change of bound CO_2 necessary for calculating the R.Q. appears as a small difference between two large readings.

(4) Analysis of the gas-phase in equilibrium with the solution containing the tissue is the basis of a further method. The application of the catharometer to the problem has been described by Slater [1926]. The apparatus is necessarily complicated.

(5) A method has been described by Fenn [1928] in which the CO_2 is absorbed by barium hydroxide solution in a special vessel arranged for the measurement of electrical conductivity. The total CO_2 liberated may be calculated from the alteration of conductivity. The oxygen is determined by Thunberg's principle.

Leaving aside method 2, which is unsuitable and, in fact, was not designed for the measurement of true respiratory quotient, a fundamental objection is found to apply to the above methods. This objection is that no allowance is made for the variations in the CO_2 chemically bound in the tissue or the surrounding solution. Any change of the acid-base equilibrium of the whole system, due to production or consumption of acids or bases by the tissue, leads to the diminution or increase of the amount of CO_2 chemically bound. Such changes are included in the figure for CO_2 production obtained by the methods 1, 4 and 5. In practice, quantitatively the most important part of the error caused by neglecting the chemically bound CO_2 is due to the lactic acid produced by glycolysing tissues. In many cases this error is so large that it makes the figure for CO_2 valueless. Many figures for respiratory quotient much above unity which may be found in the literature are obviously due to the glycolysis of the tissues examined.

From the methods described, only that of Crabtree is theoretically free from this objection. Here, however, as was mentioned above, other practical considerations limit the accuracy.

For the measurement of true respiratory quotient the only remaining principle is that in which buffer solutions almost free from CO_2 are used. The bound CO_2 present in the tissue and solution at the beginning and end of the experiment is liberated by addition of strong acid and is included in the calculation. On account of this important advantage the method has been used by many workers [Warburg *et al.*, 1914, 1921, 1924; Meyerhof, 1919; Gerard, 1927; Schorr, Loebel and Richardson, 1930; Richardson, Schorr and Loebel, 1930]. A detailed description of an experiment is given by Richardson [1929]. The solution used is buffered by phosphate instead of bicarbonate. The figures for CO_2 are obtained from manometric measurement on two pieces of tissue in two separate manometers, one with KOH, the other with acid in a side-bulb, so that the latter can be mixed with the solution and tissue in the main part of the vessel at the end of the experiment. The vessel with KOH gives the oxygen respiration, and this, together with the change of pressure occurring in the second vessel, permits the calculation of the CO_2 produced. A third manometer vessel is used to determine by acidification the initial CO_2 bound by the tissue and solution. The CO_2 content of the solution itself is determined in yet another vessel.

Valuable as the method is in certain selected cases, it suffers from an important defect which applies to all the methods where the CO_2 is determined from different pieces of tissue, already described under headings 1, 2 and 3. This is the implicit assumption that the extent of respiration, *i.e.* the volume of O_2 consumed per hour per unit weight of tissue, is exactly the same in two slices of the same tissue. Although the effect of such a difference on the respiratory quotient is not usually very serious, it is, nevertheless, desirable to remove the source of error. This is particularly the case when tissues with small respiration are concerned, and prolonged experiments are, therefore, necessary.

Another difficulty is due to the uncertainty of the amount of CO_2 retained by the tissue and phosphate during the period of temperature-equalisation preceding the experiment. During this period a portion of the respiration CO_2 is retained by the phosphate. In addition to this objection, in the case of glycolysing tissues an uncertain amount of CO_2 is driven off by the lactic acid formed. The most serious point is that all of these errors concern only the CO_2 without compensatory influence on the oxygen values.

With the above points in view, a method has been worked out so that the actual measurement could be made on a single piece of tissue[1], only one correction—a simple determination of preformed CO_2—being necessary. The accuracy of the results is not affected by glycolysis.

METHOD.

Principle. Both oxygen consumed and CO_2 produced are determined by readings on the same manometer. The measurement is made on thin slices of tissue [Warburg, 1926] suspended in CO_2-free Ringer solution, buffered by means of phosphate and in equilibrium with pure oxygen. The CO_2 formed in respiration is absorbed by barium hydroxide solution. The diminution in pressure which occurs is used for the calculation of respired oxygen. After a definite time, an excess of strong acid is added, and the whole of the CO_2 is given up from the barium hydroxide, phosphate and tissue. The resulting positive pressure is used for the calculation of the total CO_2 at the end of the experiment.

This figure, corrected for the CO_2 content at the beginning of the experiment, gives the amount of CO_2 formed in respiration during the experimental period.

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[1] The method described was worked out before that of Meyerhof and Schmitt [1929] came to our notice. Since their method, though based on similar principles, is solely concerned with muscle and nerve, the practical details in the two methods differ in many important points.

Apparatus[1]. The vessel (Fig. 1) used is attached by means of the ground joint to a Barcroft manometer. The total volume is about 20 cc. and is determined by weighing with mercury. The central portion *A* contains a piece of tissue suspended in 2 cc. phosphate-Ringer solution. The annular trough *B* contains 0·5 cc. of a cold-saturated solution of barium hydroxide. The side-bulb *C* contains 0·3 cc. of 2·5 *N* HCl, which can be tipped into the main compartments and the whole contents mixed when desired.

Solutions. Stock sodium phosphate solution. An isotonic solution of sodium dihydrogen phosphate and disodium hydrogen phosphate at p_H 7·4 is prepared with the usual precautions to exclude CO_2. With the samples of dried phosphate used, the weights were 2·43 g. NaH_2PO_4 and 12·68 g. Na_2HPO_4 to 1 litre. Such a solution is about 0·109 *M* in total phosphate. This solution is stored in a burette protected from CO_2.

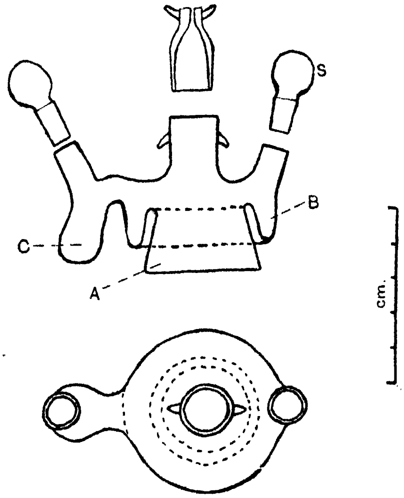

Fig. 1.

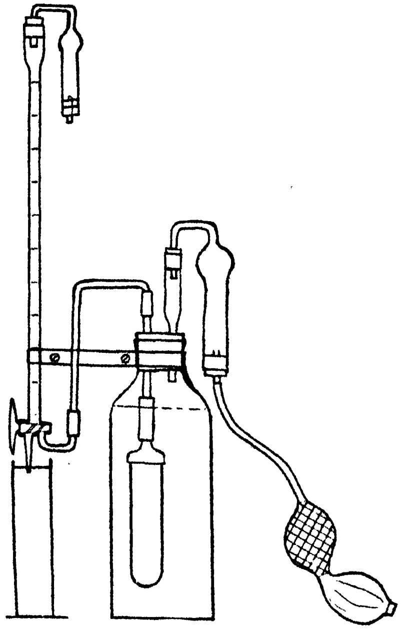

Fig. 2.

Phosphate-Ringer solution. The above solution is diluted for use by means of a suitable salt solution consisting of isotonic solutions of NaCl, $CaCl_2$ and KCl in the proportion 100: 2: 2 to which is added 10 % glucose solution to make the final concentration 0·2 %. The solution is boiled for 15 minutes and, after cooling in a stream of pure oxygen, is diluted to the original volume with CO_2-free water. The phosphate solution described above is then added. For tissues with little or no glycolysis 10 cc. phosphate per 100 cc. salt solution is sufficient. For tumours, retina and other highly glycolysing tissues a more strongly buffered solution should be used. A mixture of 40 parts to 100 salt solution has a concentration of secondary phosphate equivalent to that of bicarbonate in the solution used by Warburg. It is particularly necessary to cool the solution very thoroughly before adding the phosphate, since the mixture is supersaturated with calcium phosphate. If the vessels are perfectly clean the solution usually remains quite clear.

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[1] The apparatus was made for us by Messrs C. Dixon & Co., 27 Devonshire Street, London, W.C. 1.

Barium hydroxide solution. A cold saturated solution of barium hydroxide (*ca.* 0·3 *N*) is prepared and poured into the bottle of an automatic burette (10 cc.), fitted with a filtration candle (Berkefeld V) as in Fig. 2, CO_2-free air having been previously drawn through the whole system. The capacity of the bottle is *ca.* 500 cc. and the whole arrangement must be easily and quickly brought to the vessels when required.

Procedure. Three vessels are used in the example described (more if duplicate estimations are required. See protocol, p. 913). Another vessel, without tissue and containing the solutions only, is used as a thermobarometer to correct the readings for variations of temperature and barometric pressure during the experiment.

Preparation of the tissue. Thin slices are cut from the fresh tissue with the razor. As Warburg [1926] has shown, the thickness must not exceed a certain limiting value, in order to allow the adequate diffusion of oxygen, carbon dioxide and lactic acid in the tissue. The thin sections are rinsed for 5 to 10 minutes before the experiment by suspending in phosphate-Ringer solution through which a rapid stream of oxygen is passed. This precaution is found to give a greater uniformity in the amount of preformed CO_2 in the different slices. The oxygen used is freed from traces of CO_2 by passing through a long tube filled with soda-lime.

Preparation of the vessels. The vessels must be thoroughly cleaned overnight with strong chromic acid mixture and before use they must be washed very thoroughly to remove all traces of acid, and afterwards dried at 100°. 0·3 cc. of 2·5 *N* HCl is pipetted into the side-bulbs and 2 cc. of phosphate-Ringer solution are quickly added (the solution being run in from a pipette with two graduations) to the central portion of the first vessel. This vessel is immediately connected with its manometer and a rapid stream of CO_2-free oxygen is passed through and escapes around the loosely inserted stopper. The remaining vessels are similarly connected up, one at a time, and each of these in addition to the solutions contains an approximately equal piece of tissue. While the gas is rapidly passing, 0·5 cc. of barium hydroxide solution is measured into each vessel, the stopper *S* (Fig. 1) being momentarily removed for this purpose. The tip of the self-filling burette (Fig. 2) is protected by immersion in *N*/20 HCl until the moment of inserting into the vessel. If this operation is carried out with reasonable rapidity a constant and very low blank-value for the CO_2 content of the barium hydroxide solution is consistently obtained. The CO_2 content of the 0·5 cc. as determined by the control experiment should not exceed 6 $mm.^3$ and there is no difficulty in reducing the correction to this figure or less. The 2 cc. of boiled phosphate-Ringer solution contain nearly the same volume of CO_2, this value also being very consistently maintained throughout our experiments. Since the total volumes of oxygen and CO_2 measured are of the order of 400 $mm.^3$, the error introduced by slight variations in the correction for the solutions is negligible, provided that, as a check, their values are freshly determined for each experiment. Hence the use of more elaborate precautions in order to reduce this correction further is in general unnecessary, and would much reduce the simplicity of the technique.

The stoppers of the vessels are now pushed home, and the gas-stream is at the same moment diverted by turning the upper stop-cocks of the manometers.

The manometers and vessels are now attached to the shaking-apparatus by means of which the vessels are moved backwards and forwards in the thermostat [Warburg, 1926]. It is important that the vessels II and III, containing the tissue, should be put into the bath together so that the respiration in both follows the same course. When the vessels are shaken the barium hydroxide solution moves freely in the annular trough without any tendency to splash over into the central portion. This point is important, as the shaking must be sufficiently vigorous (excursion 5 cm.; 100 oscillations to the minute), in order rapidly to establish equilibrium of absorption of gas. Owing to the large surface presented by the barium hydroxide and the rapid movement, which entirely prevents the formation of any superficial crust of carbonate, the absorption of CO_2 is extremely efficient and control experiments have shown that it is superior to that of the usual conical vessels containing potash as absorbent.

Ten minutes after inserting the vessels into the bath the first reading of the pressure in all vessels is taken. This period is sufficient for complete equilibrium and should not be prolonged. After taking this reading, vessel II is at once removed from the bath and the acid contained in the side-bulb is thoroughly mixed with the contents by repeated tipping. The vessel is then replaced in the bath. After a further 15 minutes another reading is taken, and from the observed small increase of pressure the total preformed CO_2 in solutions and tissue is calculated. Readings of pressure in vessel III are taken at intervals of 15 or 30 minutes throughout the experiment and so the oxygen consumption is followed. After a definite time, measured from the tipping of vessel II, the acid is tipped into vessel III immediately after a reading has been taken. The large positive pressure set up is used for the calculation of the total CO_2 in vessel III at the end of the experiment.

As a check that all the bound CO_2 has been liberated the mixing of the contents of both vessels is repeated. There should be no further increase of pressure as a result of this second mixing.

At a convenient time during the above manipulations, the acid is tipped into vessel I, containing the solutions without the tissue. The slight positive pressure set up (a few mm. only) gives the correction for the CO_2 content of the solutions.

It is important that the amount of tissue used and the duration of the experiment should be so chosen that nearly the whole range of the manometer capillary (300 mm.) is used, if the greatest accuracy is to be obtained. With most tissues a period of 2–3 hours is sufficient.

At the end of the experiment the weights of the pieces of tissue are determined by removing from the vessels, thoroughly rinsing in a large volume of water, and drying to constant weight at 110°.

Calculation. From the above readings of pressure the following quantities are directly determined by simple multiplication by the vessel constants (see below). The actual exchange is determined from vessel III:

(*a*) total oxygen consumption;

(*b*) total CO_2 present at the end of the experiment;

readings (*b*) have to be corrected for the following:

(*c*) from vessel II the total CO_2 present at the beginning of the experiment;

(*d*) from vessel I, the CO_2 contained in the solutions used.

By subtracting (*d*) from (*c*) the amount of CO_2 originally present in the tissue, together with that formed by respiration during the initial period of 10 minutes, is given. The weights of the tissues being known, this correction can at once be applied to (*b*), its amount being directly proportional to the weights of the tissues. It is advisable that this correction should not exceed about 10 % of the whole volume of CO_2 in vessel III—this is always the case if the experimental period and weights of tissues are suitably selected.

The corrected value of (*b*) is the respiratory CO_2 during the period of the experiment. This value divided by (*a*) gives at once the respiratory quotient. The method of calculation for a typical experiment is given in detail in the protocol at the end of the paper.

Notes.

Vessel constants. The actual volume of gas given off or consumed ($mm.^3$) is calculated by multiplication of the pressure change observed (mm. Brodie fluid) by a constant whose value depends on the volume of the vessel and the nature of the gas. The calculation of the vessel constant (k) from the volumes of the liquid and gas (V_F and V_G $mm.^3$) at a temperature $T°$ (absolute) is [Warburg, 1926]

$$k = \frac{V_G \frac{273}{T} + V_F . \alpha}{10{,}000},$$

where α is the Bunsen solubility coefficient of the gas given off or consumed.

The solubility of oxygen, which is in any case small, may be considered as being the same in Ringer solution as in the solutions used in the present method. The same is not true of the CO_2, the solubility of which is diminished by the acidity and salt concentration of the solution obtained after mixing with acid. Since no value was available in the literature, the solubility was determined for this solution by the "first saturation method" of Austin *et al.* [1922], using the manometric Van Slyke apparatus for the estimation. The value found ($\alpha_{CO_2} = 0{\cdot}517$ at 38°: mean of four determinations) is inserted in the above equation for calculation of the vessel constants.

Accuracy. As both oxygen and carbon dioxide are determined manometrically on the same piece of tissue, the error in the actual readings (± 1 mm.) is negligible when the pressure is of the order of 250 mm. of Brodie's fluid. The error is, therefore, determined by the error in the corrections for preformed CO_2 due to tissue and solutions. The error due to variation in the CO_2 content of the solutions themselves has already been discussed. Provided the stated precautions in pipetting are observed, this error does not exceed 1 %, as we have satisfied ourselves by a large number of duplicate estimations extending over several months. In practice, therefore, the accuracy depends on the agreement between the preformed CO_2 per unit weight in the duplicate pieces of tissue (vessels II and III). The process of oxygenation of the tissue before the experiment tends to make this correction more uniform, but it is evident that the CO_2 present in the tissue and that formed by respiration in the first 10 minutes of temperature equalisation cannot be controlled as accurately as can the amount of CO_2 present in the solutions. On account of the removal of the more serious errors of previous methods, this correction becomes the determining factor in the accuracy of the present technique. However, it must be remembered that this quantity is itself a correction term the magnitude of which should not exceed about 10 % of the total CO_2. Consequently, if the error in the correction is as high as 20 %, the final error introduced will be only 2 %.

From the above considerations, the greatest error to be expected in the value of R.Q. is 0·02. A better agreement has been observed in a large number of experiments on the same tissue. These observations are included in a forthcoming paper (Part II). It is customary, in the literature of the methods discussed in the introduction to the present paper, to find the R.Q. expressed to the third decimal place. The above considerations show that such a procedure is entirely unjustified by any of these methods.

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Protocol.

Rat-brain cortex. Adult female rat fed 24 hours before experiment.

Solution. Ringer containing 0·2 % glucose and 11 m.-mols per litre phosphate-buffer p_H 7·47. Slices of tissue rinsed for 5 mins. in oxygenated solution. Duration of experiment: 3 hrs. Temp. 38°.

Vessel no.	I	II	III	IV	V	VI
Total vol. (cc.)	21·25	22·02	20·27	22·70	20·07	22·00
Side-bulb	0·3 cc. of 2·5 *N* HCl in all					
Main part	2 cc. of phosphate-Ringer in all					
Tissue	–	+	+	+	+	+
Annular trough	0·5 cc. Ba(OH)$_2$ solution in all					

Readings. After 10 mins. in bath, readings of all manometers taken.

Acid tipped in ...	Tip	Tip	No	No	Tip	No
Pressure changes at 30′ intervals corrected for thermobarometer (mm. Brodie fluid)	+ 6	+ 14·0	– 34·0	– 25·0	+ 14·5	– 44·0
		Tip again			Tip again	
	—	+ 0·5	– 36·5	– 24·0	—	– 42·0
	—	—	– 36·5	– 25·0	—	– 41·0
	—	—	– 34·0	– 23·5	—	– 42·5
	—	—	– 33·5	– 22·5	—	– 41·5
	—	—	– 35·0	– 24·0	—	– 43·5
	—	—	Tip	Tip	—	Tip
	—	—	+ 206·5	+ 145·5	—	+ 248·5
	—	—	Tip again		—	
	—	—	—	—	—	+ 0·5
Dry weights of tissue (mg.)	—	11·79	8·01	6·16	7·19	12·54
Area of slices (mm.^{2})	—	130	130	130	90	90
Thickness (mm.)	—	0·45	0·3	0·25	0·4	0·65*
Calculation:						
h_{O_2}	—	—	– 209·5	– 144·0	—	– 234·5
k_{O_2}	—	—	1·54	1·754	—	1·693
Total oxygen consumed (mm.^{3})	—	—	**323·0**	**253·0**	—	**431·0**
h_{CO_2}	+ 6·0	+ 14·5	+ 206·5	+ 145·5	+ 14·5	+ 249·0
k_{CO_2}	1·674	1·832	1·678	1·892	1·66	1·831
Total CO$_2$ (mm.^{3})	10·6	26·6	346·5	275·5	24·1	456·0
Correction for solutions	—	10·6	10·6	10·6	10·6	10·6
Preformed CO$_2$ in tissue (mm.^{3})	—	16 or 1·36 per mg.	—	—	13·5 or 1·88 per mg.	—
Correction for tissue (mean of II and V multiplied by weight)	—	—	13·0	10·0	—	20·4
Total CO$_2$ produced in mm.^{3}	—	—	**322·9**	**254·9**	—	**425·0**
R.Q.	—	—	1·00	1·01	—	0·99
Q_{O_2} (oxygen: mm.^{3} respired per hr. per mg. dry weight)	—	—	– 13·4	– 13·7	—	– 11·4

* Although this piece of tissue was too thick, the value of R.Q. is unaffected.

CI. OXIDATIONS IN CENTRAL AND PERIPHERAL NERVOUS TISSUE.

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(Received June 14th, 1930.)

INTRODUCTION.

THE experiments described in this paper were undertaken in the hope of throwing some light on the question of the oxidation mechanisms of nerve and of brain tissue. The data at present available show that the rate of oxygen consumption by the two tissues is vastly different. There can be no doubt that the brain as a whole uses oxygen at a very much greater rate than does peripheral nerve. The literature bearing on this point has been briefly discussed in previous communications [Holmes and Gerard, 1929; Holmes, 1929, 1] and will not again be referred to here. How far these data can be accepted in a strictly quantitative sense is, of course, open to question. All the experiments on the intact brain have necessarily involved the use of anaesthesia at some stage in the proceedings, while there is no means of judging how far isolated portions of brain may be expected to behave as they would *in situ.* In the experiments on nerve, the tissue has always been isolated. Still, isolated nerves can be proved to be functionally active, and it seems reasonable to assume that the oxygen which they use can be taken as a fair measure of that demanded by them while *in situ* in the living animal. On the whole, therefore, brain is likely to be more adversely affected by experimental conditions than is nerve, and probably experiments have under-rated, rather than over-rated, the true difference between the tissues.

In the present experiments, isolated rabbit or guinea-pig nerves have been employed, and their oxygen consumption has been determined by the use of a Barcroft's differential manometer. The brain tissue was that of cats, rabbits, and mice. Sometimes chopped whole brain was used; sometimes slices of cortex were employed; the oxygen uptake was observed in the same way as was that of the nerves.

1. OXYGEN UPTAKE OF UNTREATED TISSUE.

In the first place, the oxygen uptake of cortex slices, chopped whole brain (mixed grey and white matter), pure white matter from the spinal cord and peripheral nerve, was compared (Fig. 1). In these, and in subsequent experiments mentioned in this paper, the tissue was put up in a fluid having the following composition: Ringer's solution 80 parts, $M/2$ acid potassium phosphate 20 parts, NaOH, sufficient to bring the p_H to 7·6.

It will be seen that the most rapid uptake is that of the cortex (it has been found that it matters little whether it is chopped or sliced) and that the uptake of whole, chopped brain is less. In the case of white matter from the spinal cord (obtained by exposing the cord and cutting off strips of the posterior columns with sharp scissors) affairs are very different. While rabbit cortex takes up about 1200 mm.^{3} of O_2 per g. of tissue per hour, white matter takes up only from 200 to 300 mm.^{3}, an amount not very much greater than that used by peripheral nerve. This is hardly a matter for surprise, since one has no reason, at present, to suppose that the conducting elements inside the central nervous system differ in any essential way (except in power of regeneration) from those which carry impulses to and from the periphery. With regard to the uptake of oxygen by cortex, it must be remembered that the brain tissue was bathed only in Ringer's solution. If 0·25 % glucose is added to the fluid, the uptake in 3 hours is about doubled. In life, the cells are bathed in a fluid containing glucose, and in all probability therefore, from this cause alone, their uptake *in vivo* is much greater than these experiments indicate.

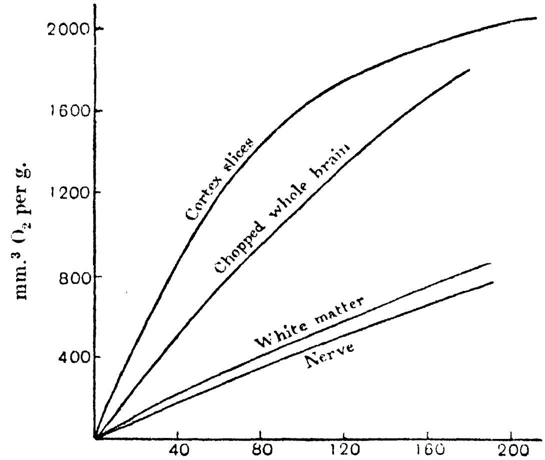

Fig. 1. Rabbit.

The results obtained with grey matter agree on the whole with those of Loebel [1925] (allowing for the fact that he used rat-cortex, and worked at 40°), while those for nerve tally well with Gerard's results [Holmes and Gerard, 1929] and with those of Sherif [1929]. It has been found that there is a very marked difference in the rate of oxygen consumption of brain tissue of different species, in the sense that smaller animals give tissue with a larger uptake. Loebel's values for rat-brain are rather higher than those here reported for rabbit (even allowing for the fact that he worked at a higher temperature). Experiment shows, on the other hand, that chopped mouse-brain (whole) takes up oxygen more quickly even than rat-cortex (*e.g.* over 1600 mm.^{3} per hour). Dixon and Elliott [1929] on the other hand, report much lower values for ox-brain.

2. OXIDATION MECHANISMS.

Attempts to investigate some of the oxidation mechanisms involved have naturally followed the lines laid down by the work of Keilin [1929][1].

A series of preliminary experiments with "Nadi" reagent left no doubt that grey matter gave a much more active indophenol oxidase reaction than did either white matter or peripheral nerve. Vernon [1911, 1912] had previously investigated the indophenol oxidase activity of various tissues, among them brain. He made his experiments quantitative by comparing the intensity of colour formation by various tissues after a given time. He observed that the intensity of the reaction varied inversely with the size of the animal. He does not seem to have employed nerve, nor to have differentiated in the brain between grey matter and white. In all cases he found that the most active reaction was given by heart muscle, and that cerebral cortex occupied a high place on the list.

Work on quantitative lines was undertaken in the present circumstances as follows. The oxygen uptake of the washed tissue, ground and suspended in buffered Ringer's solution, to which *p*-phenylenediamine was added, was observed in a Barcroft's apparatus. Without added *p*-phenylenediamine, the O_2 consumption of the suspensions was almost nil. Rabbit, mouse, ox and cat tissues were used at different times, and all gave similar results.

Cat tissues were the most convenient, since from these animals fresh tissue can be obtained in adequate amounts; they have been used to construct the curves in Fig. 2. Keilin's [1929] instructions were followed in preparing the tissue emulsions, the volumes of fluid being proportionately reduced to suit the smaller amounts of tissue. In each case, to provide a basis for comparison, 1 cc. of tissue suspension was pipetted into the cup of the Barcroft's apparatus, and a similar amount, measured with the same pipette, was introduced into a crucible, and dried at 100°. The crucible was weighed, ignited, and re-weighed, the difference giving the amount of organic matter present. The results are expressed as mm.^{3} of oxygen per 100 mg. of organic matter.

This experiment leaves no doubt that the indophenol oxidase of grey matter is much more active than that of white matter, or of peripheral nerve. The ratio of the total uptakes in this particular case is grey: white: nerve = 26·2: 4·3: 1. The difference is very much greater than that shown by the total oxygen uptakes of the three tissues, though the latter is obviously in the same direction. Since, according to Keilin, the function of indophenol oxidase is to oxidise cytochrome reduced by the tissue dehydrases, it seemed possible that the distribution of the pigment in the three tissues might follow similar lines. To investigate this, a rabbit was anaesthetised with ether, the chest opened, and cannulae tied into the aorta and right ventricle. The pulmonary artery was clamped, and the animal perfused through the aorta with Ringer's solution kept in a reservoir at 37°, the fluid escaping through the cannula in the right ventricle. (The purpose of the perfusion is, of course, to remove haemoglobin.) When about 4 litres of fluid had been run through, the perfusion was stopped, and the brain, portions of white matter from the spinal cord (posterior and lateral columns), and the sciatic nerves were removed. The three tissues were examined for cytochrome by means of a microspectroscope by Mr R. Hill. He reported that, in the cortex, there was about half as much cytochrome as in yeast; that is, much more than in skeletal muscle, but less than in heart muscle. White matter from the cord contained $\frac{1}{4}$ to $\frac{1}{5}$ as much as cortex; he could not state with certainty that any of the pigment was present in the nerves. The comparison is necessarily rough, and is based on measurement of the thickness of the slices of tissue which give absorption spectra of equal intensity. It is interesting that the amounts of cytochrome in cortex and white matter are approximately in the same ratios as the intensity of the indophenol oxidase activity—about 4 to 1 in the one case and 6 to 1 in the other.

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[1] See also review by Dixon [1929].

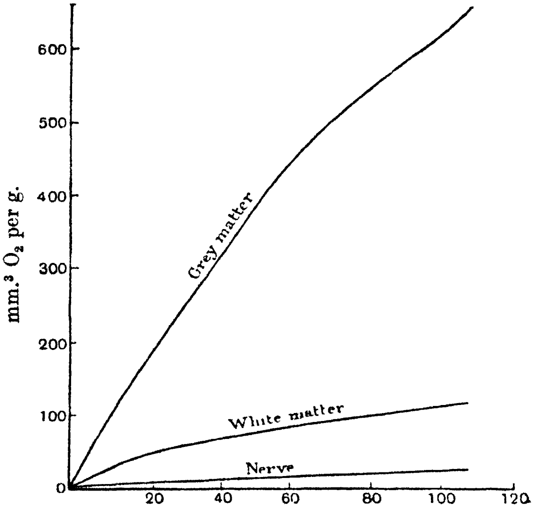

Fig. 2. Cat.

Since nerve is relatively so deficient in indophenol oxidase and cytochrome, it would be of interest to see if this fact could be correlated with its incapacity to oxidise lactic acid, a feature of its metabolism which has been previously reported [Holmes and Gerard, 1929]. Thunberg states that lactic acid will donate hydrogen to methylene blue in the presence of nerve under anaerobic conditions; it seemed, therefore, possible that methylene blue might act as a carrier of hydrogen, in the presence of oxygen, and enable lactic acid to be oxidised, thus replacing the absent oxidase system. Nerve was therefore put up in a Barcroft's apparatus with 0·5 % lithium lactate, in buffered Ringer-phosphate (p_H 7·6) containing methylene blue $M/2500$. Some experiments showed no increase in oxygen uptake in the presence of methylene blue and lactate, others an increase so slight that it cannot be accepted as significant.

3. DEHYDRASE SYSTEMS.

The fact that nerve tissue will reduce methylene blue in the absence of oxygen [Thunberg, 1923; Sherif, 1929] shows that it possesses dehydrase systems, and Thunberg reports that certain substances—glutamic acid, ketoglutaric acid, succinic, fumaric, and lactic acids—are capable of acting as hydrogen donators to methylene blue. Sherif finds that neither the presence of glucose nor of galactose effects any decrease in the reduction time.

Herter [1905] showed that methylene blue was reduced by brain tissue *in vivo*, and was re-oxidised again if the brain was exposed to the air after the death of the animal. Szent-Gyorgyi [1924] found that succinic acid acted as a hydrogen donator to brain tissue *in vitro*. He was able to show that, in the case of brain (unlike that of other tissues), the rate of oxidation of succinic acid was not limited by the rate of activation of oxygen. It has already been remarked that, in the present experiments, methylene blue was found to have no effect on the oxygen uptake of nerve. Table I shows some results obtained during the course of the present work with grey and white matter of ox-brain.

Table I.

	Reduction times in mins.	
	Grey matter	White matter
Control	13½	70
$M/50$ Lithium lactate	7	24
$M/100$ Glucose	6	48
$M/100$ Galactose	10	70

It is quite evident, from these figures, that the dehydrase mechanisms of grey matter, like the indophenol oxidase, are far more active than are those of white matter.

Since grey matter consumes oxygen so readily, and since its oxidation mechanisms seem to act so much more intensely than those of white matter or nerve, it is of interest to see what information is available as to the substrates which the three tissues are able to use.

It is well known that cortex, or whole brain, causes the rapid disappearance of lactic acid, provided oxygen is available [Warburg *et al.*, 1924; Holmes and Holmes, 1925]. It is also known that the tissue can convert glucose to lactic acid with great rapidity. From Table I it will be seen that the reduction time of methylene blue by ox-cortex is about equally rapid in the presence of glucose, as it is in that of lactic acid. To decide whether glucose was oxidised as such, or was first converted to lactic acid, advantage was taken of the fact that fluoride would prevent the formation of lactic acid by brain tissue [see Ashford and Holmes, 1929].

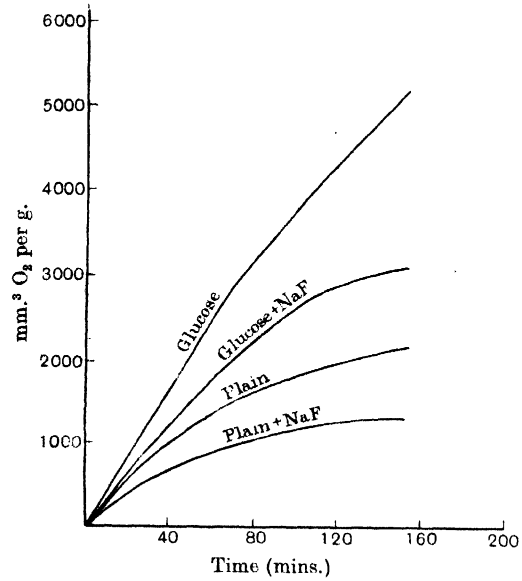

Fig. 3. Mouse.

The present experiment was arranged as follows. Equal amounts of chopped brain tissue (rabbit and mouse) were put up in each of four Barcroft's apparatus[1]. The cups of the first contained plain Ringer's solution, buffered with phosphate to p_H 7·6. The second contained a similar solution plus 0·25 % glucose, the third, as well as the glucose, 0·01 M sodium fluoride, and the fourth 0·01 M sodium fluoride, but no glucose. The curves of oxygen uptake are given in Fig. 3. It will be seen that the extra oxygen uptake due to the glucose is very greatly inhibited by the fluoride. There is also some inhibition of uptake

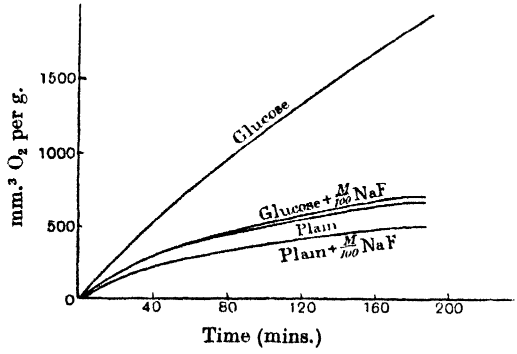

Fig. 3 *a*. Washed mouse brain.

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[1] I have to thank Mr C. A. Ashford for assistance with these experiments.

by the fluoride in the case of the tissue to which no glucose was added. This presumably indicates the inhibition of lactic acid formation from some precursor already present in the tissue. To test this point, the experiment was repeated with washed tissue. Washing very much decreases the oxygen uptake of chopped brain, and the uptake is restored by adding glucose. Fig. 3 *a* shows that this restoration is largely prevented by 0·01 *M* NaF. At the same time, fluoride still has some effect on the reduced uptake which goes on in the absence of glucose, suggesting that perhaps here it exerts an effect on the metabolism of other substances besides lactic acid—a very probable happening, in view of the findings of other workers.

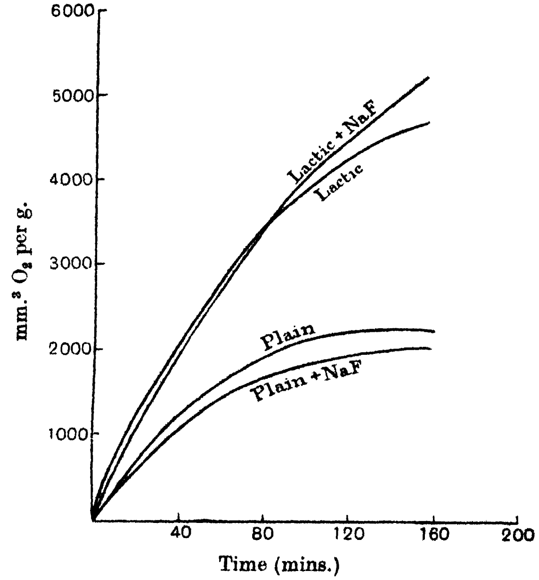

Fig. 4. Mouse.

It may be remarked that from previous experience [Ashford and Holmes, 1929] a complete inhibition of lactic acid formation by 0·01 *M* NaF was not to be anticipated; the figures previously reported indicated an inhibition of about 80 %.

If now, instead of glucose, lithium lactate be used in the above experiment, there is no inhibition of oxygen consumption: the "lactate" and "lactate and NaF" curves are almost identical (Fig. 4). It seems safe, therefore, to conclude that glucose must be converted into lactic acid before it can be oxidised by the grey matter.

As has previously been reported [Sherif and Holmes, 1930] the only effect of the addition of glucose to peripheral nerve is to prolong the period during which a nerve is able to take up oxygen at an approximately linear rate. Even this effect is usually only discernible during the course of an experiment deliberately prolonged for many hours. The same appears to be true of white matter from the cord, though the point has not yet been very fully investigated; it can, however, be said with certainty that in the case of such white matter there is no marked increase in oxygen uptake in the presence of glucose, such as is invariably obtained with grey matter in similar circumstances. It has been shown [Holmes and Gerard, 1929] that there is no disappearance of lactic acid from peripheral nerve when the tissue is kept in oxygen. It is, therefore, to be expected on all these grounds that fluoride has no influence on the oxygen uptake of nerve. Fig. 5 shows that this expectation is realised.

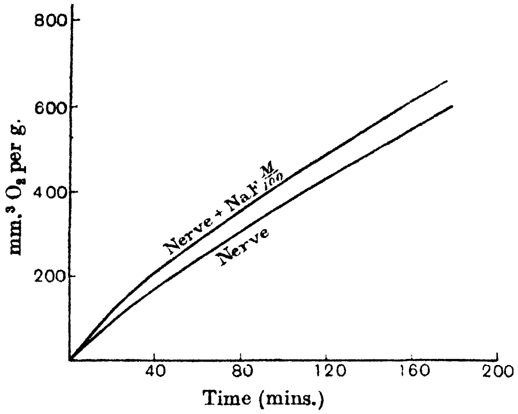

Fig. 5. Guinea-pig nerve.

4. OXIDATION OF LACTIC ACID BY "CENTRAL" WHITE MATTER.

Most of the facts so far elicited seem to suggest that white matter from the central nervous system behaves very much like peripheral nerve, a finding which at least fits in with anatomical and histological conceptions. It is, therefore, disconcerting to find that white matter incubated aerobically seems to possess some power of causing the disappearance of lactic acid. Its performance in this respect is a very poor one compared with that of grey matter; it seems, however, that it must be accepted as real.

The results of a number of experiments are given in Table II. The amounts of tissue available when rabbits or cats were employed was small, and the experimental error correspondingly large (in the neighbourhood of 10 %). The final experiment, however, in which ox-brain was used and ample tissue was available, seems to put the matter beyond doubt.

The white matter, in most of these experiments, was placed in a cup of a Barcroft's apparatus and shaken in a thermostat at 37° for 3 hours, the oxygen uptake being measured at the same time. The tissue is much more friable than is peripheral nerve, and at the end of the experiment is, for the most part, broken up; nerve, of course, in similar circumstances, remains intact. An experiment with finely chopped nerve did not, however, show any fall in lactic acid, so that apparently the phenomenon is not dependent upon the mechanical disintegration of the tissue.

Table II.

	Lactic acid: mg. per 100 g. fresh tissue						
	Initial	Final	Change	O_2 absorbed (mm.^{3})	Duration of exp. (hours)	Temp. 0°	Tissue
1*	108	88	− 20	—	2½	37	Cat's cord
2†	102	40	− 62	885	3	37	Rabbit's cord
3	93	109	+ 16	781	3	37	,,
4	107	115	+ 8	845	3	37	,,
5	119	81	− 38	751	3	37	,,
6	134	102	− 32	830	3	37	,,
7	83	84	− 1	566	3	37	,,
8	118	110	− 8	794	3	37	,,
9	116	109	− 7	575	3	37	Cat's cord
10	102	72	− 30	—	3	37	Rabbit's cord
11‡	153 (*a*)	120	− 33	—	3	37	Ox-brain
	154 (*b*)	121	− 33	—	3	37	,,
Average	116	96	− 20	753	—	—	

* Tissue kept in a tube in O_2. All remainder shaken in air.

† Extraction with trichloroacetic acid used for estimation. All remainder worked up by technique described by Holmes and Gerard.

‡ "*a*" and "*b*" duplicate samples, each of 3 g.

5. EFFECT OF WASHING NERVE AND BRAIN TISSUE.

Whatever the materials may be which are responsible for the oxygen consumption of nerve, they are not easily washed away. There is very little difference between the oxygen uptake of nerve that has been soaked in Ringer's solution for 4 hours and one kept in a moist chamber for the same period (Fig. 6). The observations of Meyerhof and Schmidt [1929] on the R.Q. of resting nerve suggest that fats are being oxidised, and this, perhaps, fits in with the foregoing observations.

In contrast to this, soaking the chopped brain tissue for 4 hours in Ringer's solution reduces the oxygen uptake enormously (Fig. 6): in contrast to nerve, therefore, either the brain depends chiefly on freely diffusible substrates, or its oxidising mechanisms are damaged by this treatment. To try to decide between these possibilities, two mice were killed, their brains chopped and sampled, and the chopped tissue soaked for 4 hours in Ringer's solution. The oxygen uptake of the washed tissue was very small, but was very much increased by the presence of 0·25 % glucose in the fluid (compare Fig. 3 *a*). In another experiment, it was found that 0·5 % lithium lactate had an even more pronounced effect. Clearly, in brain as in many other tissues, washing removes substrates, but leaves oxidising mechanisms intact.

That lactic acid is not the only substance oxidised by brain tissue is clear from a consideration of the following experiments.

(*a*) 2 g. of chopped rabbit brain were shaken in a Barcroft's apparatus[1] at 37° in air for 276 minutes. During this time, they consumed 5393 mm.^{3} of oxygen. Another 2 g. sample of the same lot of tissue gave an initial value for lactic acid of 2·53 mg. The oxygenated sample contained at the end of the experiment 0·41 mg. 2·13 mg. had therefore disappeared.

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[1] Apparatus specially constructed to deal with large amounts of tissue, to be described later in reporting other work.

Supposing this to have been oxidised, it would have required 1555 $mm.^3$ of oxygen: 3838 $mm.^3$ of oxygen are therefore "surplus" and must have been used for oxidising other substances. It may be objected that there is no proof that lactic acid was not formed and oxidised during the course of the experiment, so that, in reality more disappeared than is indicated by these figures. We have previously shown, however, that the anaerobic increase in lactic acid is small [Holmes and Holmes, 1925; Ashford and Holmes, 1929]: there is very little precursor available in the tissue, and 2·53 mg. is nearly, if not quite, a maximum value. At the most, an extra 0·5 mg. of lactic acid might have appeared.

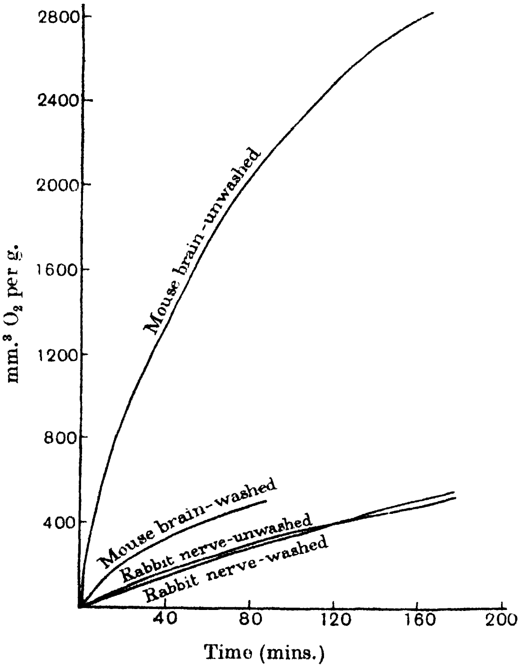

Fig. 6. Mouse brain and rabbit nerve: effect of washing.

(*b*) Six mice were starved for 12 hours. Three of them were then given 4 units each of insulin (Burroughs Wellcome) subcutaneously. After $1\frac{3}{4}$ hours, these three all showed severe hypoglycaemic symptoms. Both groups of mice were then killed, their brains excised, and the two groups of brains separately chopped and sampled. 100 mg. of tissue were then taken from each group, and the oxygen uptake observed (Fig. 7). The remainder of the tissue was used for lactic acid determinations, which gave the following values: normals 1·148 mg. per g.; insulin 0·560 mg. per g.[1]

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[1] I have to thank Mr C. A. Ashford for performing these estimations.

It is obvious that the oxygen consumed by the brains of the insulin-treated animals is less than that consumed by the normals. It is also plain that there is a very definite oxygen consumption still taking place even though there is very little lactic acid available.

It would seem, reviewing the evidence that has so far been brought forward, that we have to do with two very different types of metabolism in the brain and in the purely conducting parts of the nervous system. Such a conclusion only bears out many already well-known facts of physiology, such as the great sensitivity of the brain, and the relative insensitiveness of nerves to oxygen lack. We must assume for the present that these differences are characteristic of the nerve cell, or perhaps of the synapse on the one hand, and of the conducting elements on the other. The rate of oxygen consumption of conducting elements is of the same order, whether they be central or peripheral. Nerve cannot oxidise lactic acid, either at rest or during activity [Holmes, Gerard and Solomons, 1930]. "Central" white matter can do so, but only to a limited extent. Carbohydrate disappears from resting nerve if oxygen is available, but, even if it is oxidised, it can account for only a part of the observed oxygen consumption [Holmes and Gerard, 1929].

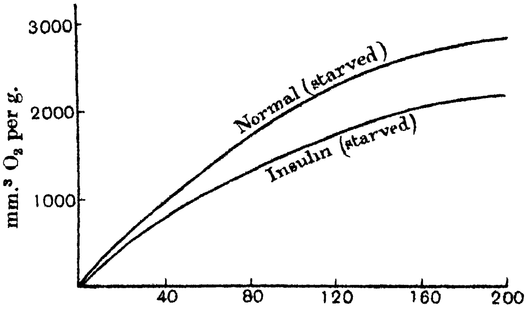

Fig. 7. Mouse.

In grey matter, replacing, or perhaps superimposed upon, a metabolism of this type, there appears to be another of a more intense kind. Oxygen is used far more freely, and the substance oxidised is certainly, in great part, lactic acid, though there is clear evidence that other substrates contribute a very appreciable quota.

The tissues dealt with in these experiments have been medullated nerve and nerve cells from the mammalian central nervous system. It is worth recalling that both the heat production [Hill, 1929] and the oxygen consumption [Meyerhof and Schultz, 1929] of non-medullated nerve are far greater than those of medullated nerve, and that the carbohydrate content [Holmes, 1929, 2] of the former is many times greater than that of the latter. No cytochrome could be detected by the writer in non-medullated nerve ganglion.

SUMMARY.

1. It has been shown that the various types of tissue that compose the central nervous system in mammals have different rates of oxygen consumption, and that the activity of grey matter is in this respect far greater than that of white matter, whether the latter forms part of the central system or is taken from a peripheral nerve. The term grey matter necessarily includes without discrimination both nerve cells and synaptic junctions.

2. The distributions of indophenol oxidase and of cytochrome run (roughly) parallel both to each other and to the rate of oxygen consumption of the different types of tissue.

3. The greatly increased oxygen uptake which grey matter displays in the presence of glucose is dependent upon the conversion of the glucose to lactic acid, and disappears if this conversion is prevented by fluoride.

4. Besides lactic acid, there are certainly other substrates responsible for some of the oxygen uptake by brain tissue; and as these substrates are not easily removed by washing, they are probably not carbohydrate in nature.

5. Carbohydrate is probably not responsible for any considerable part of the oxygen uptake of nerve, but this statement must carry the reservation that little or nothing is known of the part played by the carbohydrate fraction of the cerebrosides.

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CII. SELECTIVE FERMENTATION. I.
ALCOHOLIC FERMENTATION OF GLUCOSE, FRUCTOSE AND MANNOSE MIXTURES.

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(*Received May 27th, 1930.*)

THE knowledge of the chemical reactions involved in alcoholic fermentation and related glycolytic processes has been augmented in recent years by numerous researches on the later stages leading to the final products, carbon dioxide and alcohol. Although the individual enzymes forming the complex catalytic system "zymase" have not been separated, nor their scope, specificity, mode of action, and other properties definitely established, future findings may be expected to conform with our general conception of the mechanism of fermentation. Comparatively little is known of the primary reactions leading from carbohydrate to structures of three carbon atoms. The observation of Harden and Young coupling the evolution of carbon dioxide with the phosphorylation of a quantitatively correlated amount of sugar is the bridge between the two stages of alcoholic fermentation. Analogies occur in the carbohydrate metabolism of animal cells.

The most striking feature of glycolysis, its high stereochemical specificity, remains to be explained. A number of investigators, Dubrunfaut [1847], and especially Bourquelot [1886] and Slator [1908], demonstrated that the "zymohexoses," glucose, fructose and mannose are fermented at about equal rates when offered singly to yeast, but from a mixture like invert sugar, glucose is preferred to fructose. Gayon and Dubourg [1890] were the first to observe that some Sauterne wine yeasts behaved differently and preferred the laevorotatory moiety of invert sugar. The selectivity of dried yeast (zymin) and yeast juice (Buchner's zymase) usually parallels that of the fresh yeast from which they are prepared, but in some cases Fernbach, Schoen and Mori [1927] observed a reversion of selectivity. Fernbach and Schiller [1924] noted that culturing in alkaline media will influence the selective power of a "glucose"-yeast in the direction of increased fructose fermentation. The laws of these phenomena and of similar ones observed in other sugar mixtures will offer a clue to the mechanism of fermentation.

The speed of fermentation is independent of the sugar concentration within wide limits, and depends only on the concentration of the yeast. As the final step of alcoholic fermentation, the evolution of carbon dioxide and alcohol, is common for all fermentable sugars, it is not surprising that the formation of the end-products from a mixture should be controlled by the amount of carboxylase present. Carbon dioxide is released at a slower rate from the mixture than the sum of the rates in separate fermentation; the rate of fermentation is barely greater for the mixture than that of the faster fermenting component (cf. Table II, last column). The fermentation of glucose and fructose was treated quantitatively by Willstätter and Sobotka [1922]. The selectivity of a yeast is expressed by a factor $K_{G/F} = \frac{\ln y_0 - \ln y}{\ln z_0 - \ln z}$, where y_0 and z_0 are the initial concentrations of glucose and fructose and y and z their concentrations at any given time. It was found that the average factor was $K_{G/F} = 2{\cdot}94$ for Munich Loewenbräu yeast. This factor changed to 1·89 when the yeast was cultured on a fructose medium. Hopkins [1928; cf. Ivecovich, 1930], using the same formula, found $K_{G/F}$ about 1·8 for an English top brewery yeast; this figure was unaffected by culturing on fructose.

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[1] Isadore Hernsheim Research Fellow, 1929.

In view of the great variations of enzymic content with type and strain, we determined the selectivity of various yeasts in the present study. We found 2·10–2·21 for a bottom brewery yeast (New York Lager), 2·46 for American baker's yeast, 2·98 for a top brewery yeast (New York Ale), and 4·63 for *Saccharomyces Marxianus*. We tried to verify the preference for fructose in two strains of Sauterne yeast which we obtained from the Versuchs-Anstalt für Brauwesen in Vienna and from the Institut für Gärungsgewerbe in Berlin. However, they showed a preference for glucose. Culturing the Vienna strain on fructose reduced $K_{G/F}$ from 4·0 to 2·6 and subsequent cultures on grape juice reached $K_{G/F} = 2{\cdot}33$, which still signifies a preference for glucose. This limited adaptation to fructose resembles the behaviour of Munich brewery yeast. The $K_{G/F}$ of the New York Lager yeast was not reduced by culturing on fructose; it is analogous in this respect to Hopkins's brewery yeast which was unchanged by fructose treatment. $K_{G/F} = 2$ seems to be the lower limit of fructose adaptation. This limit was not reached by the Berlin strain which showed $K_{G/F} = 2{\cdot}8$ to 2·9 when cultured on fructose. The yeasts which were transferred from fructose to maltose media reached $K_{G/F} = 4{\cdot}0$ and 3·6 and the Berlin yeast also reached $K_{G/F} = 3{\cdot}85$ when removed from an active fermentation of saccharose before the glucose component had disappeared.

Relation of selective fermentation to saccharase and maltase.

According to recent investigations of Weidenhagen [1930] the multiplicity of saccharase, maltase, raffinase and related enzymes can be reduced to one α-glucosidase and one β-fructosidase. As these enzymes are instrumental in the formation of fructose and glucose from higher saccharides, we surmised a correlation between these specific hydrolysing enzymes and the analogous specific zymases. However, treatment of yeast as devised by Willstätter, Lowry and Schneider [1925] to increase or decrease the saccharase content failed to change the selectivity for glucose-fructose fermentation. This selectivity was also unaffected by the addition of large quantities of purified saccharase.

S. Marxianus contains fructosidase but no glucosidase, as it does not hydrolyse or ferment maltose; nevertheless, it has a decided preference for glucose over fructose.

Fermentation of mixtures containing mannose. The average $K_{G/M}$ was 3·8 with wide variations for the Lager yeast, although the rate of fermentation for the two aldoses was practically equal under the experimental conditions. Even fructose was preferred to mannose at a rate of $K_{F/M} = 1{\cdot}39$. The value of 2·73 for $K_{G/F}$ calculated from the two mannose factors tallies with those obtained experimentally in glucose-fructose mixtures.

EXPERIMENTAL.

The specific rotation of the sugars used was verified and $[\alpha]_D = -93{\cdot}6 + 0{\cdot}64\ (t^\circ - 20)$ [Laborde, 1913] was used for the temperature correction of fructose. The original brewery yeasts contained 75 % water on the average. All the other yeasts were cultured in synthetic media containing 4 % of the respective sugar besides 0·25 % KH_2PO_4, 0·25 % peptone, 0·025 % $MgSO_4$, 7 aq and 0·01 % $CaSO_4$ [Willstätter and Steibelt, 1921] and isolated after a number of 2–3 day passages by centrifugation (average moisture = 75 %).

The fermentations were carried out at room temperature. 50 cc. of a 20 % yeast suspension were mixed with 75 cc. of a 10 % sugar solution and samples withdrawn at the times given. The progress of fermentation was followed in the van Iterson-Kluyver apparatus [van der Haar, 1920] with 1 cc. of the fermentation mixture containing 60 mg. sugar. The theoretical amount of carbon dioxide from this amount of sugar with the necessary corrections for absorption, temperature and pressure is 15·6 cc. at 20° and 760 mm. The figures given in Table I under CO_2 represent volumes measured in % of the theoretical total. Fermentation in the samples was stopped by addition of a few crystals of sulphosalicylic acid and rapid centrifugation. The clarified supernatant liquid was analysed polariscopically and by the Hagedorn-Jensen method in Hanes's modification or by the Bertrand method. Corrections were applied for the yeast blank when necessary. The reduction of the sugar mixtures was interpolated from the reduction values of their constituents.

Table I gives examples for each yeast used. $K_{G/F}$ was computed by using the decimal logarithms of the sugar concentrations as given in the preceding columns in % of total sugar originally present.

A synopsis of our experiments is given in Table II. The time necessary for evolution of half the theoretical amount of carbon dioxide was noted in the Kluyver apparatus. The relative speeds of fermentation for glucose, invert sugar and fructose were referred to that of fructose = 1. These figures are recorded in the last two columns of Table II. The deviation from unity is, with very few exceptions, less than 10 %, in accordance with previous investigations.

Table I. *Determination of $K_{G/F}$ (ratio glucose fermentation/fructose fermentation) in various types of yeast.*

Yeast and temp.	Time (min.)	Sugar (actual %)	Optical rotation $l = 2$ dm.	Specific rotation	Not fermented in % of total original sugar			$K_{G/F}$	CO_2 %
					Total sugar	Glucose	Fructose		
1. Lager	0	6·30	− 2·26°	− 17·92°	100·0	49·6	50·4	—	—
30°	60	5·04	− 2·37	− 23·51	80·0	36·5	43·5	2·08	—
	90	4·40	− 2·38	− 27·07	69·8	30·0	39·8	2·13	—
	120	3·74	− 2·32	− 31·01	59·4	23·9	35·5	2·08	—
								2·10	
2. Ale	0	6·48	− 2·02	− 15·6	100·0	53·2	46·8	—	—
21°	120	5·57	− 2·31	− 20·75	86·0	42·7	43·3	2·85	—
	210	4·66	− 2·62	− 28·1	71·9	32·1	39·8	3·12	—
								2·98	
3. Baker's	0	6·55	− 2·99	− 22·8	100·0	48·65	51·35	—	—
20°	130	3·37	− 3·04	− 45·1	51·5	17·25	34·25	2·56	30·3
	215	1·40	− 1·89	− 67·7	21·3	3·85	17·45	2·35	54·5
								2·46	
4. *S. Marx.*	0	6·14	− 2·57	− 20·9	100·0	49·55	50·45	—	—
21·5°	150	4·58	− 3·14	− 35·25	74·6	29·5	45·1	4·65	18·6
	255	3·64	− 3·43	− 47·1	59·35	18·75	40·6	4·49	30·1
	335	2·96	− 3·49	− 58·8	48·35	11·35	37·0	4·76	39·8
								4·63	
5. Vienna	0	6·31	− 2·59	− 20·5	100·0	50·5	49·5	—	—
Saut. on	70	5·73	− 2·64	− 23·0	90·8	44·4	46·4	2·17	—
gr. juice	115	5·20	− 2·75	− 26·45	82·4	38·3	44·1	2·40	—
17·5°	160	4·53	− 2·81	− 31·0	71·8	31·1	40·7	2·48	—
	245	4·00	− 2·70	− 33·75	63·4	26·35	37·05	2·25	—
								2·33	
6. Berlin	0	5·60	− 2·62	− 23·4	100·0	48·0	52·0	—	—
Saut. on	95	4·54	− 2·81	− 30·9	81·0	34·75	46·35	2·82	16·0
fructose	145	3·60	− 2·93	− 40·7	64·3	23·3	41·0	3·04	34·0
20°	197	3·08	− 2·77	− 44·7	55·0	18·4	36·6	2·73	42·3
								2·90	
7. Berlin	0	5·74	− 2·60	− 22·7	100·0	48·35	51·65	—	—
Saut. on	50	4·66	− 2·99	− 32·1	81·4	34·3	47·1	3·74	14·7
sucrose	85	3·50	− 2·99	− 42·75	61·1	19·9	41·2	3·93	26·3
20°	130	3·01	− 3·01	− 50·0	52·6	14·1	38·5	4·19	40·4
	190	1·99	− 2·46	− 62·0	34·7	6·05	28·65	3·53	53·8
								3·85	
8 *a*. Lager	0	6·46	− 2·44	− 18·9	100·0	50·9	49·1	—	—
+ saccharase	90	3·78	− 2·56	− 33·85	58·5	23·8	34·7	2·19	32·7
	135	2·28	− 2·32	− 50·9	35·3	10·2	25·1	2·41	50·0
21°								2·30	
8 *b*. Lager	0	6·40	− 2·49	− 19·45	100·0	50·55	49·45	—	—
(control)	90	3·79	− 2·62	− 34·55	59·2	23·8	85·4	2·25	33·3
21°	135	2·26	− 2·22	− 49·15	35·3	10·6	24·7	2·25	51·2
								2·25	
							Mannose	$K_{G/M}$	
9. Lager	0	6·40	+ 4·08	+ 31·85	100·0	46·0	54·0	—	—
26°	60	4·70	+ 2·61	+ 27·75	73·5	25·9	47·6	4·55	—
	90	3·70	+ 1·87	+ 25·2	57·8	16·55	41·25	3·80	—
	120	2·90	+ 1·37	+ 23·85	45·3	11·35	33·95	3·02	—
								3·8	
						Fructose	Mannose	$K_{F/M}$	
10. Lager	0	6·38	− 4·70	− 36·8	100·0	49·1	50·9	—	—
26°	60	4·40	− 3·00	− 34·2	68·9	32·1	36·8	1·30	—
	90	2·44	− 2·10	− 30·8	53·5	23·2	30·3	1·44	—
	120	2·54	− 1·44	− 28·4	39·8	16·35	23·45	1·42	—
								1·39	

Table II. *Ratio of glucose fermentation/fructose fermentation in separate and mixed solutions.*

Yeast	Exp.	Remarks	$K_{G/F}$ (for mixture)	Ratio for rate of separate ferm. gluc./fruct.	Ratio invert sugar/fructose
Lager	No. 1.	Orig. (12. vii. 1929)	2·10	1·06	1·03
		Orig. (22. xi. 1929)	2·18	—	—
		Orig. (4. xii. 1929)	2·21	0·91	1·07
		Grown on fructose	2·50	0·98	1·11
	No. 8 *a*.	Saccharase added	2·30	0·98	1·32
	No. 8 *b*.	Control	2·25	1·02	1·20
		Enriched in saccharase	2·24	0·77	1·00
		Poor in saccharase (acid treatment)	2·13	1·00	—
Vienna Sauterne		Grown on maltose	3·98	0·98	0·98
		Grown on fructose I	3·12	1·06	1·00
		Grown on fructose II	2·60	1·04	1·01
		Grown on glucose	2·89	0·95	0·95
	No. 5.	Grown on grape juice	2·33	1·15	—
		Recultured on maltose	3·97	1·00	1·03
Berlin Sauterne		Grown on fructose	2·80	0·87	1·16
		Grown on maltose	3·62	1·00	0·97
		Grown on saccharose	2·80	0·89	0·98
	No. 7.	Grown on saccharose (interrupted)	3·85	0·98	1·19
	No. 6.	Grown on fructose	2·90	0·99	1·01
Ale	No. 2.	Orig. (5. xii. 1929)	2·98	1·21	0·85
Baker's	No. 3.	Orig. (10. xii. 1929)	2·46	1·00	0·99
S. Marxianus	No. 4.	Grown on saccharose	4·63	0·98	1·40

The saccharase determination of the Lager yeast yielded a saccharase value [Schneider, 1929] of 0·0044. By the stimulating treatment of Willstätter, Lowry and Schneider [1925] this value was more than doubled; by acid treatment it was reduced to 0·0017. The $K_{G/F}$ of these yeasts did not differ from the values for the original yeasts.

In Exp. 8, 166 mg. purified "Invertase scales" of a saccharase value 0·325, comprising the saccharase content of more than 10 g. yeast, were added to 100 cc. of a Lager yeast fermentation. No change of $K_{G/F}$ was noted.

SUMMARY.

1. The factor $K_{G/F}$ for several types of yeast was determined. All types preferred glucose to fructose at a rate of 2 : 1 or higher.
2. This factor is subject to changes depending on the culture medium used.
3. No correlation was found between selective fermentation and saccharase or maltase content.
4. The selective fermentation of mixtures of mannose plus glucose and mannose plus fructose was studied.

We wish to thank Mr Leo Wallerstein for samples of yeast and of concentrated invertase.

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CIII. THE HEAT-STABILITY OF THE (ANTI-DERMATITIS, "ANTI-PELLAGRA") WATER-SOLUBLE VITAMIN B_2. II.

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THE following experiments form a continuation of those recently published by Chick and Roscoe [1930] which indicated that vitamin B_2 in yeast or yeast extracts, though relatively stable to temperatures at 100–120° in acid solution, was sensitive to these temperatures if the reaction were alkaline. Thus, at p_H 5·0–3·0 there was a 50 % loss in vitamin B_2 content after heating for 4–5 hours at 123°; at p_H 8·3–10·0 a loss of between 75 and 100 %. When heated for 2 hours at 90–100° in faintly acid solution no loss in potency was detected; at p_H 8·3, on the other hand, about one-half was lost and even when maintained for 10 days at room temperature (summer) in a weakly alkaline solution there was a loss of about one-third of the original strength. Vitamin B_2 was estimated by the method previously described [Chick and Roscoe, 1928].

These conclusions are not in accord with those of Reader [1929, 1930], who found vitamin B_2 able to withstand heating for 1 hour at 120° at p_H 9·0, or of Narayanan and Drummond [1930], who state that no appreciable destruction of this vitamin takes place when yeast products are heated for 1–3 hours at 110–120° in a solution containing 10–15 % barium hydroxide.

This divergence is of more than usual interest, for the stability of vitamin B_2 to heat and alkali is important in the chain of arguments indicating the existence of Reader's third water-soluble B vitamin. This new factor is stated to differ from vitamin B_2 in being sensitive to heat and to alkali.

CRITERION USED AND METHODS EMPLOYED IN ESTIMATION OF VITAMIN B_2.

In the experiments of Reader and of Narayanan and Drummond, as well as in our own, the criterion used in the detection and assay of vitamin B_2 was the power to promote or restore growth in young rats receiving diets complete in every other respect. It is obvious that a more satisfactory criterion than growth, because more specific, would be the cure of the dermatitis (? rat-pellagra) which develops in young rats after several weeks of vitamin B_2 deficiency. Such experiments are by nature unsuited for quantitative work owing to the irregularity of these symptoms as regards their nature, time of onset and degree of severity. Nevertheless, it was felt that they might provide useful confirmation of previous work [see Chick and Roscoe, 1930, p. 111] and in this hope the following experiments have been carried out. The results have confirmed our previous findings in demonstrating that the curative property for ratdermatitis, present in yeast extract equally with the power to restore growth of rats on diets lacking only vitamin B_2, is sensitive to high temperatures in alkaline solution.

Technique of the curative tests.

The increase in body weight of young growing rats is extremely sensitive to deprivation of vitamin B_2. When these animals are placed on diets from which this vitamin is absent, increase in weight usually ceases within a few days and it is only in exceptional circumstances (as for example in the condition known as refection [Fridericia *et al.*, 1927; Roscoe, 1927]) that an approach to even subnormal growth is manifested. The onset of the characteristic dermatitis is, however, more irregular. Sometimes it is marked after 6–8 weeks of deprivation; on the other hand, it may take 20 or more weeks to develop. The distribution, character and severity of the skin affection also vary greatly [Chick and Roscoe, 1928]. The curative tests described below were carried out on a collection of "negative" control rats belonging to different litters which had been maintained for various periods on the standard "$-B_2$" diet for the express purpose of studying these skin lesions. As will be seen from the details set out in the accompanying table, these varied considerably, but in selecting animals for the tests care was taken that, as far as was possible, the doses of the materials compared were administered to animals whose condition was similar.

The symptoms usually present when treatment began were the following. Sunken eyes with blood-stained secretion and inflamed eyelids, inflamed skin, bare of fur, surrounding the eyes giving a characteristic "spectacled" appearance. The fur was thin and poor, especially over the head, neck and chest; in severe cases the bald patches often developed into raw areas spreading round the axilla and down the forearm. The wrists of the forepaws were almost invariably stained with the bloody discharges from eyes and nostrils, as the result of incessant rubbing. The fur of the lower part of the abdomen was also stained reddish brown with what appeared to be blood from the urine. These stains, however, unlike those on the wrists, have repeatedly failed to give the characteristic tests for blood or blood-pigments.

The stains in both places are alike the first symptoms to disappear, often within a day or two after treatment with an adequate curative dose. Next in order, the eyes usually recover their normal prominent position and the skin begins to assume a healthy texture and appearance. Lastly, new fur makes its appearance, 2–3 weeks being needed before the coat may be described as normal.

A set of skin appearances differing somewhat from the above was frequently encountered in our earlier work, but now rarely occurs [Chick and Roscoe, 1928, p. 795]. These include (1) inflammation of the tips of the ears, which become cheesy in texture and, during the cure, shed their dead epidermis in flakes, (2) an oedematous inflammation of the digits of the paws, which become bright red in colour. On one occasion these symptoms made their appearance while cure of a rat (564 ♀) with general dermatitis was being attempted with an inadequate dose of material containing vitamin B_2, suggesting the possibility that a small, though inadequate, amount of vitamin B_2 in the diet was necessary for demonstration of these more florid skin symptoms of vitamin B_2 deficiency [see also Sherman and Sandels, 1929].

It is worth noting that these particular symptoms of vitamin B_2 deficiency have been less frequent since the use of more highly purified caseinogen in the basal diet, although the failure to grow and the development of a generalised dermatitis have been more constant. The suggestion that these florid symptoms are induced by a less complete deprivation of vitamin B_2 is confirmed by the fact that, on one occasion, a rat (456 ♀) exhibiting these symptoms, but otherwise in good condition, was slowly cured when treated with a yeast extract which had been autoclaved at 120° for 4 hours in alkaline solution, this being the only instance where material so treated has, in our experience, exhibited curative properties for the dermatitis caused by vitamin B_2 deficiency.

An analogous instance is probably that of rats receiving diets almost, but not entirely, deprived of the antineuritic vitamin B_1. In the course of several weeks such rats will often develop the characteristic paralysis due to vitamin B_1 deficiency, whereas those suffering complete deprivation usually die in 3–4 weeks from collapse without manifestation of any nervous affection [Hofmeister, 1922, 1, 2].

Vitamin B_2 material used in the present tests.

A 0·01 % acetic acid extract was prepared from washed brewer's yeast, sample XXIV, similar to that made from yeast XII, used in the experiments of Series B (Table II) described in the previous paper [Chick and Roscoe, 1930]. One portion (*a*) of the extract was acidified to p_H 2·5, and a second portion (*b*) made alkaline by addition of sodium hydroxide to p_H 10·3, the hydrogen ion determinations being made with a hydrogen electrode. After heating for 4 hours at 119° the p_H of (*b*) (which had fallen to 9·6) was adjusted to about 3·0 for storage. That of (*a*) remained at 2·5. Both solutions were adjusted to their original volume and in the following tests of the curative power of the two solutions all doses are reckoned as the equivalents of the original yeast (dry weight).

No tests were made with the unheated material, as it was felt that no accurate quantitative results could be expected from such curative tests and that it would be sufficient to determine the relative curative value of the materials heated in acid and alkaline solution respectively, comparing the results obtained with those of the previous work when growth was the criterion. Ten tests were made, six rats being treated with material (*a*) and four with (*b*).

Results of the curative tests.

Yeast extract autoclaved in acid solution.

Of six rats treated with doses of this material, three received a daily dose equivalent to 0·5 g. yeast. They all showed marked improvement in a week, and normal appearance of skin and fur was attained in 2½ to 3 weeks (rats 645, 574, 624). Simultaneous with the improvement in the skin condition there was immediate resumption of growth, the average weekly increase in body weight being 10–20 g.

With smaller doses of the same material the result was not so dramatic. Rat 589, which had a severely affected skin after 16 weeks' deprivation of vitamin B_2, received the equivalent of 0·33 g. yeast daily for 4½ weeks. At the end of this time the body weight had increased by 38 g. (from 66 to 104 g.), and the skin and fur, though much improved, were not quite restored to a normal condition. Rats 664 and 564 received the equivalent of 0·16 g. yeast daily. This proved inadequate for complete cures, though the skin condition was improved and growth was resumed.

Yeast extract autoclaved in alkaline solution.

Four rats received this material in doses equivalent to 0·5–1·0 g. yeast daily, for periods of from 3 to 5 weeks; they showed no improvement and in three cases the skin lesions became definitely worse. For example, rat 599, with only a moderate skin affection, received the equivalent of 1·0 g. yeast for 23 days, during which time the symptoms became progressively more severe. Bare, desquamating patches of skin developed round the eyes, and on the neck and shoulders bald areas appeared, which later became raw and inflamed. Diarrhoea also occurred and there was a slight loss in weight (10 g.).

Rat 565 received a dose equivalent to 0·5 g. yeast daily for 21 days during which the skin symptoms became steadily worse, and there was slight loss of weight. Increasing the dose to the equivalent of 1·0 g. yeast was of no use, but, when this was replaced by extract autoclaved in acid solution equivalent to 0·5 g. yeast, a swift improvement took place. In 10 days the skin had assumed a healthy appearance, new fur was growing, the blood-stained discharges from nose and eyes had discontinued and the diarrhoea, which had developed during the preceding period, also disappeared. There was an increase in weight of 27 g. in the 10 days.

Rats 616 and 635 received the material treated in alkaline solution in the equivalent of 0·5 and 1·0 g. yeast respectively. Rat 616 became definitely worse during the 27 days of its treatment and lost 9 g. in weight; rat 635 on the larger dose maintained weight and continued with symptoms unchanged for 5 weeks. The subsequent cure of these two rats took place with material kindly provided by Dr Reader. This material was a solution of marmite autoclaved at 120° for 1 hour at p_H 9·0 and was found by Dr Reader to be effective in curing the characteristic dermatitis due to deficiency of vitamin B_2, but

Heat-stability of vitamin B_2 in yeast extract (XXIV). Autoclaved at 119° *for* 4 *hours. Curative tests. Vitamin B_1 provided as Peters's concentrate.*

p_H of extract when heated	Dose, expressed as equivalent of dry yeast g.	Rat no. and sex	Time on "– B_2" diet. Weeks	Symptoms: general condition	Body weight g.	Average weekly increase in body weight during treatment g.	Result of treatment
2·5	0·5	574 ♀	16	Eyes badly affected, bloody discharge from nose and eyes. Fur very poor on chest	72	16	Rapid cure. 1st week, eyes and skin became healthy and new fur began. Skin and eyes normal in 2½ weeks
2·5	0·5	624 ♀	12	Skin symptoms severe. Eyes sunken, secreting, "spectacled." Bloody discharge also from nose. Fur very poor, thin and "stringy" over face and neck. Abdomen stained	47	14	Rapid cure. 1st week. Eyes nearly normal, posture also. 2nd week. All symptoms disappeared. 3rd week. Normal fur
2·5	0·5	645 ♀	6	Skin symptoms slight but animal very weak and apparently moribund. Abdomen stained	42	21	Rapid cure. Coat appeared normal in 9 days
2·5	0·33	589 ♀	16	Skin symptoms severe. Eyes "spectacled"; left nearly closed. Fur poor and stringy on face and neck. Bloody discharge from nose and eyes. Abdomen stained	66	9	Slow cure. Eyes improved and stains on fur from discharges disappeared in 3 weeks. New fur growing and coat becoming normal in 4½ weeks
2·5	0·16	604 ♀	12	Eyes "spectacled." Paws blood-stained. Fur poor and "stringy" over face and neck. Abdomen stained. Condition miserable	55	6	Very slow cure. Symptoms arrested in 1st week. Stains on abdomen disappeared in 3rd week, skin and eyes not normal after 4 weeks
2·5	0·16	564 ♀	21	Skin symptoms severe. Eyes "spectacled," nostrils inflamed, bloody discharge. Fur "stringy," bald patches round ears, raw patches round anus and vagina. Abdomen stained. Condition miserable	50	7	Marked improvement in 1 week, ears and forepaws desquamating. Skin round vagina dry and healthy

10·3–9·6	1·0	599 ♂	17	Symptoms slight. Eyes sunken, "spectacled." Bloody discharge from nose. Fur thin on face. Abdomen stained	81	Lost 10 g. in 23 days	No cure. Symptoms became worse in 2–3 weeks. Bare, raw, desquamating patches developed round eyes and on left side of neck and shoulder. Skin round nose and lips became swollen and cracked, area of inflamed skin developed round anus. Animal very miserable. Killed
10·3–9·6	1·0	635 ♂	10	Skin symptoms severe. Eyes sunken, "spectacled." Bloody discharge from nose. Fur "stringy." bald patches round ears. Nose tip inflamed	49	2·5	No cure. Condition remained almost unchanged except for onset of diarrhoea during 5 weeks' treatment. Treated with Dr Reader's preparation of marmite. Marked improvement in 1 week. In 3 weeks coat almost normal, animal in normal health, increase in weight 72 g.
10·3–9·6	0·5 later 1·0	565 ♂	15	Skin symptoms not severe. Eyes sunken and inflamed. Bloody discharge from nose. Diarrhoea	88	Lost 15 g. in 22 days	No cure. Condition became worse. In 3 weeks raw, bald patches were spreading from the axillae down the forearms. Mouth sore and inflamed. Condition of animal miserable. Treated with acid-autoclaved material equivalent to 0·5 g. yeast. Rapid cure. In 10 days nose healing, new fur growing everywhere, increase in weight 27 g.
10·3–9·6	0·5	616 ♂	13	Skin symptoms slight and incipient. General condition fair	56	Lost 9 g. in 27 days	No cure. Condition rapidly grew worse. In 3 weeks inflamed, bare patch of skin developed under chin, nostrils inflamed, fur "stringy." Treated with Dr Reader's preparation of marmite. Marked improvement in 1st week, in 3 weeks nostrils healed, coat almost normal; increase in weight 56 g.

incapable of restoring growth on basal diets lacking in vitamin B_2 and provided with abundant vitamin B_1. In our experiments, not only were the skin lesions rapidly healed but immediate vigorous growth took place. The weight of rat 635, on the dose prescribed by Dr Reader, was increased by 72 g. in 3 weeks and that of rat 616, on about half this dose, by 56 g. in the same time.

This divergence would be explained if our preparation of vitamin B_1 (Peters's concentrate prepared from brewer's yeast) contained Dr Reader's third factor. We are now examining this preparation for evidence of any factor other than the antineuritic vitamin B_1 and are endeavouring to separate it, if present. The results of these researches will be published shortly. At the present time we can only report that we have failed to obtain satisfactory cures of dermatitis unaccompanied by increase in weight. Another possible explanation of the results obtained with Dr Reader's solution is that the material was originally so rich in vitamin B_2 that the residue, after exposure to 120° for 1 hour in alkaline solution, still contained sufficient in the doses given to cure the dermatitis and to stimulate growth in the rats of our experiment.

SUMMARY.

1. An extract made from washed brewer's yeast (XXIV) by boiling in dilute acetic acid, after being heated in acid solution (p_H 2·5) for 4 hours at 119° (solution *a*), retained its power to cure the skin affection in rats due to deprivation of vitamin B_2 swiftly and completely in doses equivalent to 0·5 g. yeast (dry wt.) and more slowly and less completely in doses equivalent to 0·16–0·33 g. yeast.

2. When the same extract was exposed to the same treatment in alkaline solution (p_H 10·3–9·6) (solution *b*), no curative properties could be demonstrated in doses equivalent to 0·5 g. or 1·0 g. yeast and in three out of four cases the skin affection became rapidly worse.

3. Growth of the rats was parallel with the changes in the condition of the skin; when treated with solution (*a*) growth was restored, with solution (*b*) body weight remained stationary or decreased.

In conclusion we wish to thank Dr Reader for providing the material used for the cure of rats 565 and 616, and Messrs Watney, Coombe, Reid & Co. for providing the brewer's yeast from which our extract was made.

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CIV. THE STABILITY OF WATERY SOLUTIONS OF THE OXYTOCIC PRINCIPLE OF THE PITUITARY GLAND.

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It has long been known that the substance in the posterior lobe of the pituitary gland which produces contractions of the uterus is destroyed by alkalis in the cold [Guggenheim, 1914], is stable in weakly acid watery solution, and is destroyed by boiling in strong acids [Abel and Nagayama, 1920; Dale and Dudley, 1921].

Adams [1917] showed that when weakly acid extracts were heated the oxytocic activity, measured on the guinea-pig's uterus, disappeared from the solution in such a way that its rate of disappearance at any moment was proportional to the activity present at that moment. The solutions were much more stable at p_H 3 than at p_H 5.

This paper deals with a number of determinations which have been carried out, under my direction, by my assistant, L. S. Drewell, with the object of obtaining more complete quantitative knowledge of the stability of this substance. Buffered extracts were prepared from the British standard preparation of the posterior lobe, heated for different times and assayed. The tests were carried out on the virgin guinea-pig's uterus by the method described by Dale and Laidlaw [1912], the details of the apparatus being those described by Burn and Dale [1922]. The results are comparable with, and in many ways similar to, the results obtained by Krogh and Hemmingsen [1928] in their study of the action of heat on insulin. For the sake of uniformity the notation of these authors has been adopted in the present paper.

METHODS.

Heating was carried out in ampoules of hard glass, sealed and placed in a water-bath, the temperature of which was maintained constant. This was effected either by means of a toluene regulator, or when, as in most cases, temperatures near to 100° were required, by boiling the water and maintaining its level with an overflow tube—a procedure which produced a constant temperature of about 99°.

Over a wide range of p_H it would be inconvenient to study the rate of destruction at low temperatures, since it would be necessary to keep the extracts for many months in order to get any considerable destruction. The effect of p_H on the stability has, therefore, been most completely studied at 99°. The application of these results to the stability at lower temperatures is discussed below.

The hydrogen ion concentration of the solutions was buffered where possible with NaOH and NaH_2PO_4 in different proportions [Prideaux, 1911]. Potassium was excluded from the solutions because it would have interfered with the final assay. Between p_H 3 and p_H 5·5, where the buffering of phosphates is slight, Walpole's HCl-acetate mixtures were used. For one measurement at p_H 8·5 Palitsch's boric acid-borate mixture was used [Clark, 1928].

The total concentration of phosphate, acetate or borate was $N/7$, so that these solutions were approximately isotonic with blood. The p_H of the solutions was determined colorimetrically, and in most cases electrometrically, and was found not to differ widely from the p_H calculated from the weights of the salts used in preparing the buffer. Solutions of p_H 1 and p_H 2·1 were obtained by diluting a standard extract in 0·25 % acetic acid with HCl, so that the final concentration of HCl was $N/10$ and $N/100$. Similar solutions in N HCl, N NaOH and $N/10$ NaOH were taken to represent p_H 0, p_H 14, p_H 13. In these very acid and alkaline solutions the initial concentration of the pituitary extract was 1 unit per cc. In all the other experiments it was 2 units per cc.

The measurements of p_H were made at room temperature, whilst most of the experiments were carried out at about 100°. It is improbable that this circumstance introduced any considerable error since the p_H of phosphate buffers [Walbum, 1920], acetate buffers [McIntosh and Smart, 1920; Clark, 1928], and strong acids and bases are said to be practically unaffected by changes of temperature. In all cases the solutions were made faintly acid for testing.

In the case of the observations between p_H 3 and p_H 7·25 extracts were made by boiling the standard powder in the buffer solution for 10 minutes, cooling and filtering. The filtrate was heated to a known temperature for a known time and its activity then determined by comparison with a standard extract in 0·25 % acetic acid. The assumption was made that the extraction was complete, and a small correction was applied for the loss of activity during the initial 10 minutes' boiling. In the case of the observations at p_H 11·1 it was possible in the light of more complete knowledge to adopt a more satisfactory procedure. The extraction was carried out in a solution of acid sodium phosphate. A portion of this extract was kept as a standard solution and another portion was treated with a suitable quantity of NaOH, maintained at a constant temperature for a definite time and then faintly acidified with HCl, cooled, made up to a known volume and tested.

The course of the reaction.

If Adams's [1917] conclusions as to the course of the reaction are correct, the velocity of the change may be measured, like that of the destruction of insulin, in terms of a constant k which is equal to the rate of fall per hour of $\log_{10} C$ (where C is the concentration of the active principle).

The fact that k remains constant has been confirmed by carrying out two or more experiments at each of seven different values of the p_H in which the time of heating was varied. These results are shown with others in Fig. 1. The values of k showed no regular tendency either to increase or decrease and the variations which occurred were no larger than might have been expected.

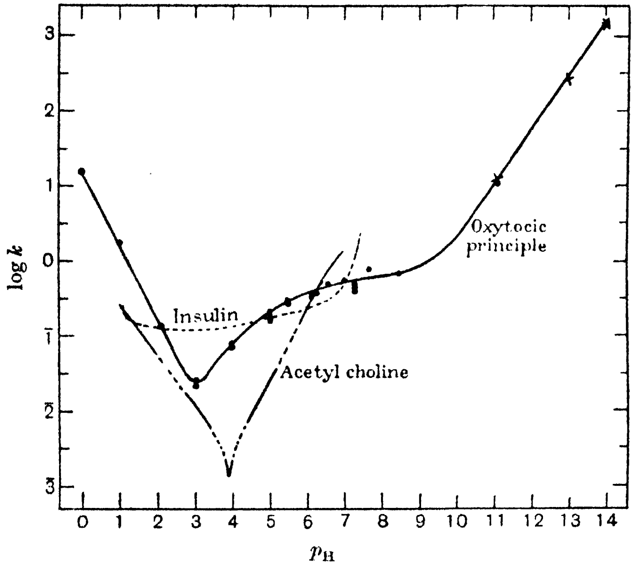

Fig. 1. Abscissae—p_H. Ordinates—log k—where k is rate of destruction at 99°. Dots represent direct observations. Crosses represent results calculated from observations at 25°.

In order to determine k under any particular circumstances it is desirable that the reaction should continue until destruction is nearly complete, since in this case the error of the assay has less effect on the result. The experiments have therefore been planned so that the percentage destruction was always over 50 and usually about 90, but it was, of course, not possible to ensure uniformity in this respect.

The effect of temperature.

The variation of the velocity of the reaction with the absolute temperature T is defined with sufficient accuracy by Arrhenius's equation

$$k_1 = k_0 e^{\frac{\mu}{2}\left(\frac{T_1-T_0}{T_1 T_0}\right)},$$

where k_1 and k_0 are the rates of destruction at absolute temperatures T_1 and T_0. Measurements have been carried out at p_H 6·55 and at p_H 11·1 and the results are recorded in Table I. In each case three estimates of the constant μ in the above equation have been obtained by combining the result of the experiment at 99° with the results of those at lower temperatures.

Table I.

Temp. °	Time of heating (hrs.)	Activity left %	k (corrected)	μ
		p_H 6·55		
99	1·33	17·6	0·503	—
80	16·0	1·39	0·111	20,900
60	42·0	11·6	0·02	20,500
50	69·2	33·8	0·0056	22,100
		p_H 11·1		
99	0·1	8·0	10·97	—
74·5	0·783	3·0	1·94	18,200
50	10·68	3·3	0·142	21,300
25	47·67	33·3	0·01	21,000

The variations in μ are irregular and are not large considering the methods used. For the purpose of calculation it is assumed that μ has a constant value of 21,300—an estimate of its value which was obtained graphically. These observations have also been taken to justify the assumption that the value of μ is independent of the p_H. The absolute value of μ is low compared with the values obtained for the spontaneous disintegration of various more complex substances [Arrhenius, 1915]. It is lower than the value obtained for the destruction of insulin—28,300 [Krogh and Hemmingsen, 1928], but it is high compared with the values met with in most chemical reactions.

The effect of hydrogen ion concentration.

The variation of k with the p_H is large and in order to get all the readings on the same curve the p_H has been plotted in Fig. 1 not against k but against its logarithm. The readings shown by dots were obtained in the boiling water-bath, the temperature of which was 99°. The readings shown by crosses were obtained at 25° and the values which would have been obtained at 99° were calculated from the data given above and plotted.

In the region of strong acidity the results fall on a straight line the slope of which indicates that the rate of destruction is directly proportional to the hydrogen ion concentration.

In the region of strong basicity the results also fall on a straight line, but in this region the rate of destruction is proportional to $[C_{OH}]^{0\cdot73}$.

The effect of variation of the p_H on the stability is at a minimum between p_H 7 and 8.

For the sake of comparison similar curves for insulin and for acetylcholine are shown. The former is taken from the paper by Krogh and Hemmingsen [1928]. The latter was obtained by combining some observations published by Hofmann [1930] with some unpublished observations which Dr K. Mathes has kindly allowed me to use.

One experiment was carried out to test the possibility that the rate of destruction was considerably affected by the amount of salt in the solution. It was found that when the concentration of phosphate at p_H 7·2 was divided by ten, the other factors remaining constant, the value of log k fell by 0·071.

It was concluded that if any salt effect is present it is small compared with the other factors and it has been neglected.

All the results are summarised in Fig. 2.

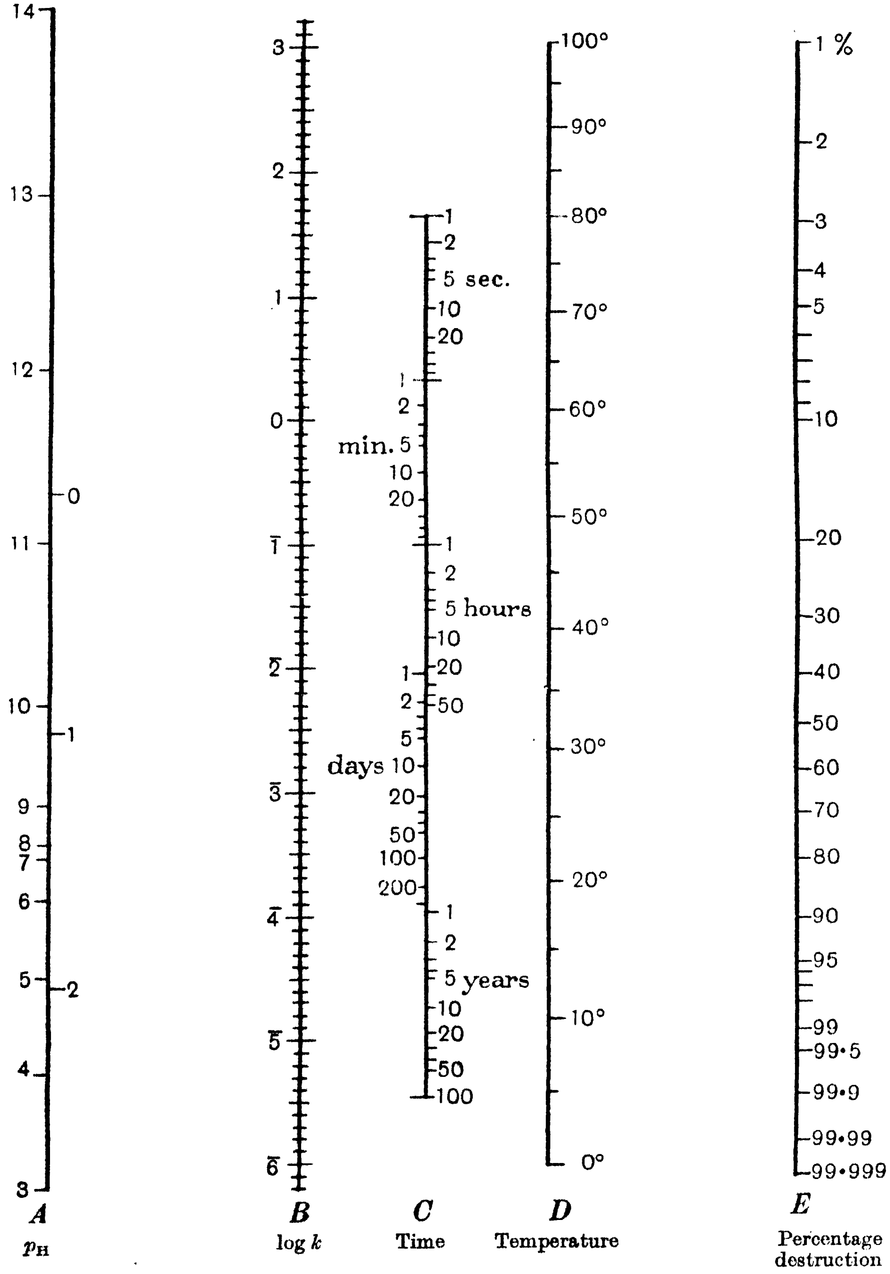

Fig. 2. Log k is determined on scale B by aligning the p_H on scale A with the temperature on scale D. The percentage destruction is obtained on scale E by aligning log k on scale B with the time on scale C.

The use of this nomogram is most readily explained by taking an example. The p_H of the standard extract in 0·25 % acetic acid is about 4 [Kamm *et al.*, 1928]. If a thread be stretched from p_H 4 on scale A to 100° on scale D it will be found that when this solution is boiled $\log k = \bar{2}\cdot 9$. By stretching the thread through $\bar{2}\cdot 9$ on scale B and 10 minutes on scale C it is found that when this solution is boiled for 10 minutes about 3 % of the oxytocic principle is destroyed. Similarly, if this solution is kept for a year at 0° the loss of activity is about 5 % and would probably not be detected. This last conclusion has been confirmed.

It should be emphasised that these results were obtained with extracts of a powder which contained all the substances present in the posterior lobe of the pituitary which are soluble in water, but not in acetone. It is possible that preparations that are either more or less pure may give different results. It has, however, been found that the stability of the preparation known as "pitocin" or "oxytocin" [Kamm *et al.*, 1928], at p_H 7 and 99°, is identical, within the error of the method, with that of the oxytocic substance in the comparatively crude extracts used in the present experiments.

SUMMARY.

The rate of destruction of the substance in watery extracts of the standard preparation of the posterior lobe of the pituitary gland, which causes contraction of the guinea-pig's uterus, has been measured at different hydrogen ion concentrations and at different temperatures. The results are summarised in a nomogram (Fig. 2) which gives the amount of destruction to be expected when a solution of any given p_H is heated for any given time at any given temperature.

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CV. SYNTHESIS OF THE *i*-HYDROXY-ASPARAGINES.

By ALBERT CHARLES CHIBNALL.

A NOTE ON THEIR DISSOCIATION CONSTANTS.

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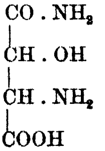
The important part played by acid amides in the protein metabolism of the plant is too well known to require emphasis: these substances have long attracted the attention of plant chemists on account of the relative ease with which asparagine and glutamine—the only amides which have yet been found in natural products—can be isolated from plant extracts. It is perhaps less well known that the amount of these two amides which can be obtained in crystalline form from any given plant extract is always less than that which the "amide-nitrogen," as determined by Sachsse's method [1873], suggests should be present. Two explanations of this readily suggest themselves. In the first place the presence of other soluble plant products prevents the crystallisation of the total amount of asparagine or its copper salt. Where the amount of these other products is small, as in intensely etiolated lupin seedlings, Schulze [1880] obtained a yield of 90 % of crystalline asparagine; but in less intensely etiolated plants, or in the normal green plant wherein the other products predominate, the yield is always low and seldom exceeds 30 %. In the second place, it may well be that amides other than asparagine or glutamine are present in the plant, and that these have so far escaped detection because they possess different solubility properties from the other two amides. Schulze admitted this alternative, which appears the more probable from Vickery's [1924] recent work on the water-soluble nitrogenous materials of green alfalfa plants. Vickery's results suggest that the possible unknown amides were similar to asparagine and glutamine, *i.e.* they were monoamides of monoaminodicarboxylic acids. As on theoretical grounds it would seem possible that one of these might be a hydroxyasparagine, the synthesis of the *i*-hydroxyasparagines was undertaken so that future search for one of the possible new amides in plant products could be guided by a knowledge of the chemical and physical properties of these substances.

The synthesis presented in some ways the same difficulties which confronted

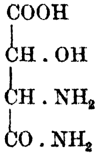
Piutti [1888] when he worked out the constitution and synthesis of the α- and β-asparagines. These arose chiefly from the fact that the action of hot alcoholic ammonia on the diethyl ester of aspartic acid did not give the expected diamide, but a ring product, "aspartimide," which was afterwards shown by Fischer to be the diamide of 2 : 5-diketopiperazine-3 : 6-diacetic acid. In the present instance it was found that the action of hot alcoholic ammonia on the diethyl ester of the *anti*-acid led to considerable decomposition, and no definite substance could be isolated from the resulting dark brown oil. The synthesis of the diamide was, however, readily accomplished by the method of Fischer and Königs [1904], who showed that the diethyl ester of *l*-aspartic acid and fluid ammonia at room temperature reacted to produce the diamide of *l*-aspartic acid (yield about 50 %) and "aspartimide." This diamide on standing with cold aqueous baryta gave a good yield of *l*-β-asparagine.

i-para- and *i-anti-*Hydroxyaspartic acids were synthesised from maleic acid without difficulty by Dakin's [1921] method and reacted with fluid ammonia to give the diamide in good yield, together with other products which probably correspond to "aspartimides." When the diamide was allowed to stand with cold aqueous baryta partial hydrolysis occurred, with the formation of both the α- and β-hydroxyasparagines.



$$\begin{array}{l} CO.NH_2 \\ | \\ CH.OH \\ | \\ CH.NH_2 \\ | \\ COOH \end{array}$$

β-Hydroxyasparagine
(1)



$$\begin{array}{l} COOH \\ | \\ CH.OH \\ | \\ CH.NH_2 \\ | \\ CO.NH_2 \end{array}$$

α-Hydroxyasparagine
(2)

The α- and β-hydroxyasparagines are readily differentiated by means of their copper salts. The β-amide (1) gives a normal copper salt containing one equivalent of the metal, whereas the α-amide (2), having the hydroxyl group contiguous to a carboxyl group, takes up two equivalents, indicating that the hydrogen of the hydroxyl has been replaced by metal. Similar abnormal salts are given by hydroxyaspartic acid itself, by *iso*serine, and, as is well known, by malic and tartaric acids. Confirmatory evidence of the position of the amide group in these two amides is given by the biuret reaction. Schiff [1900] showed that the colour developed depends on the number of carbon atoms separating the NH_2-groups. The amide which the copper salt suggests is α-hydroxyasparagine gives a red-violet (oxamide type) colour, showing that the NH_2-groups are attached to adjacent carbon atoms, while the β-hydroxyasparagine gives a blue-violet (malonamide type) colour, showing that the NH_2-groups are connected through a chain of three carbon atoms. Similar colour reactions are given by α- and β-asparagine respectively.

The α-hydroxyasparagines crystallise with 1 H_2O in well-defined stars and long tapering needles, somewhat resembling glutamine. They are readily distinguishable from *l*-β-asparagine. The crystals of the β-hydroxyasparagines however are anhydrous, and under the microscope exhibit rhombic hemihedral forms which are indistinguishable from those of *l*-β-asparagine. Unlike the latter they exhibit no tendency to form large crystals on slow evaporation of an aqueous extract, a difference which is still apparent when a mixture of the two is allowed slowly to crystallise.

With regard to the general properties of the hydroxyasparagines, the α- and β-forms, as mentioned above, are readily distinguishable by their differences in water of crystallisation, crystalline form, and copper content of the copper salts. They also exhibit different dissociation constants. No differences, however, have been noted—except perhaps slight ones in solubility—between the corresponding *anti*- and *para*-forms.

Both the α- and β-hydroxyasparagines reduce permanganate fairly readily in neutral solution, an observation which can be used to distinguish them readily from *l*-β-asparagine. They give no reaction with Fenton's reagent for tartaric acid, and their colour reactions with various phenols and sulphuric acid do not differ in any marked way from those given by *l*-β-asparagine. Like the latter, also, they have no sharp melting or decomposition point: when heated they slowly darken above 175° and decompose between 230° and 250°.

As to the possible presence of a hydroxyasparagine in plant products the following observations may be of use.

(1) With mercuric nitrate they give heavy white precipitates similar to that given by *l*-β-asparagine.

(2) The copper salts are practically insoluble in cold water, but are fairly readily soluble in boiling water in contradistinction to the copper salt of *l*-β-asparagine, which is soluble only with difficulty in boiling water. The copper salts of the hydroxyasparagines are soluble in excess of copper acetate. Boiling with excess of freshly precipitated cupric hydroxide or with copper carbonate is therefore recommended in any attempt to isolate these salts from plant extracts. Differences in the colours of these various salts which are noted below may prove, in practice, to be somewhat deceptive, as the exact shade of blue varies somewhat with the degree of fineness of the crystals.

(3) If one equivalent of aqueous baryta be added to a solution of any hydroxyasparagine and carbon dioxide passed, no precipitate of barium carbonate occurs, even on boiling, indicating the presence of either an abnormal barium salt or carbamate. Sufficient material has not been available further to investigate this interesting observation.

(4) The α-hydroxyasparagines crystallise so readily and have such a small solubility in cold water that it is improbable that they would have escaped detection had they been present to any extent in plant extracts. The β-hydroxyasparagines, however, are much more soluble in water, and have been found in certain cases to crystallise only with great difficulty in the presence of much foreign material. They might well be present in the syrupy mother liquors which are known to contain so much of the unaccounted "amide-nitrogen" of plant extracts.

(5) Plant chemists occasionally identify asparagine by its crystalline form and nitrogen content, and also glutamine by the copper content of its copper salt. It may not be out of place to call attention to the fact that for all practical purposes β-hydroxyasparagine ($C_4H_8O_4N_2$) and β-asparagine ($C_4H_8O_3N_2$, H_2O) have the same crystalline form and nitrogen content, and that the copper content of the copper salt of β-hydroxyasparagine is the same as that of the copper salt of glutamine.

EXPERIMENTAL.

i-*Hydroxyaspartic acids.* Dakin's method [1921] was followed in all essential details. Maleic acid was used as the starting material for the synthesis of bromomalic and chloromalic acids, which were then heated with strong ammonia at 100° for 10 hours to give a mixture of the *i*-hydroxyaspartic acids. These were separated by fractional crystallisation.

i-para-*Hydroxyaspartic acid diethyl ester.* 6 g. of finely powdered acid were suspended in 50 cc. of absolute alcohol and esterified in the usual way with hydrogen chloride. The ester hydrochlorides were decomposed with K_2CO_3, the ethereal solution of the esters dried with anhydrous sodium sulphate and the ether removed in high vacuum at 30°. The ester was a pale yellow viscous oil. Yield 5·5 g. or 70 % of theory. No attempt was made to purify the product by distillation *in vacuo*, as Dakin states that this leads to considerable decomposition. (Found: C, 46·1; H, 7·2; N, 6·8. $C_8H_{15}O_5N$ requires C, 46·8; H, 7·3; N, 6·8 %.)

i-para-*Hydroxyaspartic acid diamide.* 6·3 g. of the diethyl ester were placed at the bottom of a thick-walled Carius tube, which was then cooled by solid carbon dioxide and acetone. Three to four volumes of fluid ammonia were then run in and the tube sealed. The tube was removed from the bath, allowed to attain room temperature and gently shaken to mix the ester and fluid ammonia. At the end of the first day small white crystals appeared in the liquid, and at the end of the fourth day, when the tube was opened, the walls of the tube in contact with the fluid were covered with a crystalline mat. The fluid ammonia was allowed to evaporate slowly. The resulting yellow crystalline mass was scraped out of the tube, powdered, extracted twice with 40 cc. of warm ethyl alcohol to remove unchanged ester and dried *in vacuo* over solid sodium hydroxide. Yield 4·2 g. Found: N, 27·1 %; $C_4H_9O_3N_3$ requires N, 28·6 %, so that products other than the diamide had been formed. These could not be separated by extraction with warm ethyl alcohol as in the case of the corresponding *anti*-preparations, for the diamide is itself somewhat soluble in this reagent. The mixture was very soluble in cold water, but on standing for some days a small amount of crystalline diamide separated. Recrystallised from a little hot water it was obtained in well-formed cubes and rectangular plates. (Found: C, 32·5; H, 6·1; N, 28·7. $C_4H_9O_3N_3$ requires C, 32·6; H, 6·1; N, 28·6 %.) Heated in an open capillary tube it darkens slowly above 170°, shrinks at 173° and melts with decomposition and evolution of gas at 174° (corr.). The diamide reacts alkaline to litmus, and, as one would expect, gives an intense red-violet biuret reaction. It is freely soluble in warm water, and when pure is much less soluble in cold water. It is somewhat soluble in warm methyl and ethyl alcohols, only slightly soluble in the cold solvents or in acetone, and is insoluble in ether.

It gives a heavy white precipitate with mercuric nitrate, soluble in excess of nitric acid and a precipitate with mercuric chloride which dissolves on warming and reappears on cooling. It gives a small precipitate with phosphotungstic acid on standing, but none with chloroplatinic acid or with concentrated sulphuric acid. The aqueous solution on boiling slowly gives off ammonia. When 0·35 g. dissolved in 350 cc. of water was boiled gently in a flask about one-quarter of the total nitrogen was removed in about 16 hours. The solution slowly darkened and on allowing the residual liquid to evaporate slowly to a syrup in a desiccator a dark brown gum, similar to that given by the action of hot alcoholic ammonia on the ester, was obtained. The action of a mild alkali, such as baryta, results in the splitting off of one amide group, with the liberation of one-third of the total nitrogen as ammonia and the formation of a mixture of the two monoamides.

i-α-para-*Hydroxyasparagine.* 3·5 g. of the crude *para*-diamide were dissolved in a little water and placed in a Van Slyke apparatus for determining amide-N *in vacuo.* Water containing 6 g. of crystalline baryta was then added, and the total volume made up to 70 cc. At the end of 16 hours the ammonia set free was distilled off *in vacuo*, the residual liquor filtered to remove a small white deposit, which probably contained barium *para*-hydroxyaspartate, and barium removed exactly with sulphuric acid. The resulting solution was concentrated to 40 cc. *in vacuo* and allowed to stand overnight. 0·85 g. of crystalline material, consisting chiefly of tapering needles and complicated stars, was removed. On standing for a further 36 hours another 0·29 g. of similar material was obtained. The combined crystalline fractions were dissolved in 45 cc. of hot water, filtered from a small amount of insoluble residue and allowed to crystallise slowly. The resulting product, when viewed under the microscope, was seen to consist almost entirely of tapering prisms and stars, with only occasional plates of the β-amide. After two further crystallisations from water a product (0·8 g.) free from the β-amide was obtained. It crystallises from water in fine tapering prisms and stars of complicated form, and contains 1 H_2O which is only removed in high vacuum at 100°. (Found: C, 28·7; H, 5·9; N, 17·1; H_2O, 10·8. $C_4H_8O_4N_2$, H_2O requires C, 28·9; H, 6·0; N, 16·9; H_2O, 10·8 %.) It is freely soluble in boiling water, and crystallises readily from solution on cooling. At 18° it is soluble in about 125 parts of water. If a solution of the amide is first neutralised by the addition of one equivalent of sodium hydroxide and excess of a strong solution of copper acetate added, the solution turns a deep blue and becomes strongly acid to litmus, owing to the liberation of acetic acid and the formation of an abnormal salt containing two equivalents of copper. It crystallises readily in microscopic aggregates of stars and needles containing 1 H_2O, which is not readily removed at 105°. (Found: H_2O, 8·0. $Cu(C_4H_6O_4N_2), H_2O$ requires H_2O, 7·9 %.) For analysis the material was dried *in vacuo* over P_2O_5 at 100°. (Found: C, 23·0; H, 3·1; N, 13·1; Cu, 29·6. $Cu(C_4H_6O_4N_2)$ requires C, 22·9; H, 2·8; N, 13·4; Cu, 30·4 %.)

The copper salt, which can be prepared equally well by boiling with freshly precipitated and well-washed cupric hydroxide, or by the addition of copper acetate without the previous addition of sodium hydroxide, is almost insoluble in cold water, but is moderately soluble in boiling water, from which it can be readily recrystallised. The blue colour of the salt is a little paler than that of crystalline copper sulphate and is easily distinguished from that of the corresponding β-hydroxyasparagine and also of *l*-β-asparagine.

i-β-para-*Hydroxyasparagine.* The mother liquor from the isolation of the corresponding α-monoamide mentioned above was allowed to evaporate slowly in a desiccator over sulphuric acid. When the volume had been reduced to less than 5 cc. crystals appeared, and after several days the syrupy residue had set nearly solid. It was filtered at the pump and washed with a little cold water. The white crystalline mass (0·9 g.) was dissolved in 20 cc. of warm water and allowed to evaporate slowly in a desiccator. The first two crops of crystals consisted chiefly of hexagonal plates, and on repeated recrystallisation from water there was finally obtained a fraction (0·4 g.) which appeared under the microscope to be uniform, consisting of simple and complicated rhombic plates and prisms, which are indistinguishable from the corresponding forms of *l*-β-asparagine. The β-monoamide contains no water of crystallisation. (Found: C, 32·2; H, 5·5; N, 18·6; amide-N [Sachsse, 1873], 9·3. $C_4H_8O_4N_2$ requires C, 32·4; H, 5·4; N, 18·9; amide-N, 9·4 %.) The β-monoamide dissolves in about 55 parts of cold water and is freely soluble in boiling water. In the presence of each other the α- and β-monoamides crystallise out readily in their respective forms, but in the presence of other substances their solubility, especially that of the β-monoamide, is greatly increased, and crystallisation is slow. The β-monoamide and *l*-β-asparagine crystallise readily in the presence of each other in crystals that appear uniform and may be mixed. The α-monoamide also crystallises readily in the presence of *l*-β-asparagine, but the two crystalline forms are distinct, and a tendency to form mixed crystals was not observed.

The copper salt is prepared in a similar way to that of the α-amide. It separates in small amorphous spheres, which, under high magnification, are seen to consist of star-shaped aggregates of stumpy needles. It contains one equivalent of copper, and 1 H_2O which is not completely removed in an oven at 105°. (Found: H_2O, 4·8. $Cu(C_4H_7O_4N_2)_2$, H_2O requires H_2O, 4·8 %.) For analysis the material was dried *in vacuo* at 100° over P_2O_5. (Found: C, 26·8; H, 4·1; Cu, 17·8; N, 15·8. $Cu(C_4H_7O_4N_2)_2$ requires C, 26·8; H, 3·9; Cu, 17·8; N, 15·7 %.)

The synthesis of the amides of the *i-anti*-hydroxyaspartic acid was similar to that described for the *para*-acid above, consequently only points of difference in the two series and necessary analyses are recorded.

i-anti-*Hydroxyaspartic acid diethyl ester.* This acid resembles glutamic acid in that a relatively large amount of alcohol is necessary to effect its initial solution. 6 g. of the finely pulverised acid were suspended in 60 cc. of absolute alcohol, and a rapid stream of dry hydrogen chloride was passed in until the alcohol boiled. The boiling was continued for 1 hour on the water-bath, but much acid still remained undissolved. The mixture was therefore cooled, and the dry gas again passed until the alcohol boiled. The boiling was continued for another hour, when complete solution of the acid was obtained. The ester hydrochloride, though fairly soluble in alcohol, is very insoluble in cold alcohol containing hydrogen chloride. Yield of ester, a pale yellow oil, 4·7 g. or 60 % of theory. It was not distilled. (Found: C, 46·4; H, 7·5; N, 6·6. $C_8H_{15}O_5N$ requires C, 46·8; H, 7·3; N, 6·8 %.)

i-anti-*Hydroxyaspartic acid diamide.* 4·5 g. of the ester were treated with fluid ammonia. The yield of crude diamide was 3·2 g. This was purified by extracting twice with 150 cc. of boiling absolute alcohol. The material (2·5 g.) contained 28·2 % N, and was therefore practically pure *anti*-acid diamide. A small amount of this was dissolved in hot water. On standing overnight it crystallised in small cubes and irregular oblong prisms. (Found: C, 32·7; H, 6·2; N, 28·6. $C_4H_9O_3N_3$ requires C, 32·6; H, 6·1; N, 28·6 %.) Heated in an open tube it commences to darken above 170°, shrinks at 176°, and melts with decomposition at 176·5° (corr.). It gave similar reactions to the *para*-diamide.

i-*α*-anti-*Hydroxyasparagine.* 2·1 g. of the diamide were dissolved in 70 cc. of water containing 4 g. of crystalline baryta and treated as in the case of the *para*-diamide. Barium was removed, the filtrate concentrated to 20 cc. and on standing overnight 0·8 g. of crystalline material separated. Recrystallised once from warm water it was obtained in blunted needles and prisms, free from the *β*-monoamide. It contains 1 H_2O which is not removed at 105°. (Found: H_2O, 10·8; amino-N, 8·4. $C_4H_8O_4N_2, H_2O$ requires H_2O, 10·8; amino-N, 8·4 %.) It dissolves in about 95 parts of cold water and is freely soluble in boiling water. The copper salt is prepared in a similar way to that of the *para*-amide, and has a similar appearance and solubilities. (Found: C, 20·8; H, 3·6; Cu, 28·3; H_2O, 8·0. $Cu(C_4H_6O_4N_2), H_2O$ requires C, 21·0; H, 3·5; Cu, 28·0; H_2O, 7·9 %.)

i-*β*-anti-*Hydroxyasparagine.* The mother liquor from the isolation of the *α*-monoamide described above was allowed to evaporate slowly in a desiccator. Crystallisation was slow, but after some days 0·47 g. had separated and was removed. Redissolved in 10 cc. of warm water it crystallised slowly in needles and stars of the *α*-monoamide and a few plates of the *β*-monoamide. After a tedious fractionation there was finally obtained 0·1 g. of material which consisted entirely of the rhombic plates characteristic of the *β*-monoamide. It contained no water of crystallisation. (Found: C, 32·8; H, 5·6; N, 18·0. $C_4H_8O_4N_2$ requires C, 32·4; H, 5·4; N, 18·9 %.) It is readily soluble in hot water, and has about the same solubility in cold water as the corresponding *α*-monoamide. The copper salt separates in nodular aggregates like those of the *para*-*β*-monoamide, containing one equivalent of copper and 1 H_2O. (Found: H_2O, 5·2. $Cu(C_4H_7O_4N_2)_2$, H_2O requires H_2O, 4·9 %.) For further analysis the material was dried in high vacuum over P_2O_5. (Found: C, 27·0; H, 4·2; Cu, 18·0. $Cu(C_4H_7O_4N_2)_2$ requires C, 26·8; H, 3·9; Cu, 17·8 %.)

DISSOCIATION CONSTANTS.

Complete hydrogen electrode titrations of aspartic acid, hydroxyaspartic acid, *i*-*β*-asparagine, *i*-*β*-*para*-hydroxyasparagine and *i*-*α*-*anti*-hydroxyasparagine have been carried out. From the data the "titration constants" of each substance have been calculated. These values must be close approximations to the dissociation constants uncorrected for activity ($p_{K'}$) and will be assumed to be identical with them. The mean values are assembled in Table I. Following Brønsted [1923], all constants have been formulated as hydrion dissociation constants. Consequently, the subscript numeral indicates only the order in which the several constants of a substance appear on the p_H scale. If we adopt the zwitterion hypothesis of Bjerrum [1923], assignment of the various constants may be made as follows:

$p_{K_1'}$, the first carboxyl of the dibasic acids and the carboxyl of the amides;

$p_{K_2'}$, the second carboxyl of the dibasic acids and the amino-group of the amides;

$p_{K_3'}$, the amino-group of the dibasic acids.

A number of determinations of the dissociation constants of aspartic acid have appeared in the literature. Some of these, recalculated to conform to the above treatment, are included in the table. Agreement is fair. We have found only one previous determination for *β*-asparagine. The method employed was that of measurement of the rates of ester hydrolysis. The values so obtained agree well with our results by the more precise electrometric method. The constants of the remaining substances have been determined for the first time. The assembled results form an interesting contribution to the problem of the effect of substitution upon ionisation. They do not, however, cover a sufficient number of cases to make profitable a detailed analysis from this point of view. Moreover, simple effects of substitution are undoubtedly complicated, in substances such as these, by powerful electrostatic forces between ionising groups. It is clear, however, that both the introduction of hydroxyl and the substitution of the hydroxyl group in carboxyl by amide increases the hydrion dissociation of both carboxyl and amino-groups. The magnitude of these effects is reduced to about one-third as the substituent is removed from the *α*- to the *β*-position. It is interesting to compare the influence of *α*-amide formation with that of peptide formation. Comparison with the constants of a typical amino-acid and its dipeptide indicates that these effects upon the amino-group are similar in sign and in magnitude.

Table I.

	COOH	COOH	NH_2	Mol. conc.	Temp. ° C.	Author
	p_{K_1}'	p_{K_2}'	p_{K_3}'			
Aspartic acid	1·90	—	9·47	—	25	Levene and Simms [1925]
	2·08	3·94	9·85	—	—	Hopfield *et al.* [1920]
	2·08	3·66	9·85	—	—	Harris [1924]
	2·05	3·76	9·85	0·02	25	Chibnall and Cannan
Hydroxyaspartic acid	1·95	3·47	9·03	0·02	25	Chibnall and Cannan
	p_{K_1}'		p_{K_2}'			
β-Asparagine	2·18	—	8·87	—	—	Lunden [1906]
	2·02	—	8·80	0·02	25	Chibnall and Cannan
β-Hydroxyasparagine	2·12	—	8·26	0·01	25	Chibnall and Cannan
α-Hydroxyasparagine	2·31	—	7·17	0·01	25	Chibnall and Cannan
Glycine	2·42	—	9·74	—	25	Quoted by Kirk and Schmidt [1929]
Glycylglycine	3·15	—	8·10	—	25	

EXPERIMENTAL.

The technique of titration followed that of Cannan and Knight [1927]. The temperature was accurately maintained at 25°. The reference electrode was a saturated calomel half-cell in electrical contact with the hydrogen electrode vessel through a saturated potassium chloride bridge. The standard of reference was 0·1 *M* hydrochloric acid which was assumed to have a hydrogen electrode potential of 0·0643 v. at 25°. The potentials of the liquid junctions, which were established in a reproducible fashion, were ignored. A pair of palladinised gold-plated platinum wires served as hydrogen electrodes. In no observation did they differ from one another by more than 0·2 mv. The calculation of "titration constants" followed the method of Simms [1926].

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CVI. THE EFFECT OF CRYPTORCHIDISM AND OF CASTRATION ON THE CHEMICAL COMPOSITION OF RATS.

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(*Received June 25th, 1930.*)

This investigation not only aimed at ascertaining whether cryptorchidism and castration produce any change in the chemical composition of the tissues of the rat, but was also a trial of a simple method of obtaining approximate, but accurate, information about the general chemical composition of the bodies of such small animals as mice, rats or guinea-pigs. It was mainly desired, in this method, to avoid a long and difficult treatment of the whole body of the animal for analysis. In the present paper only the amount of water, solids, nitrogen and fat in the tissues of the rats was investigated.

HISTORICAL.

Reference has been made in previous papers [Korenchevsky, 1914, 1925, 1928, 1930; Korenchevsky and Carr, 1925; Korenchevsky and Schultess-Young, 1928] to the whole literature on castration and cryptorchidism. It is perplexingly contradictory. The fact which is most definitely established is that in most castrated animals and human beings an increase in the body-fat is observed when the testes are removed or are atrophied. Korenchevsky's [1930] experiments on rats confirmed this quite definitely, as well as showing that most cryptorchid animals also become obese, although to a lesser degree than the castrates.

As far as we know there is only one investigation on this subject, by Lüthje [1902, 1903], who studied the chemical composition of whole castrated animals, namely, of two castrated dogs as compared with that of their normal litter mates. Lüthje's technique was perfect, but unfortunately the number of animals used in the experiments, owing to the difficulty of technique, was insufficient for any conclusions. Nevertheless, we refer to these experiments at length, since, considered in connection with our experiments, Lüthje's results will assist in the drawing of our conclusions. The following interpretation of Lüthje's results is ours and was not suggested by him. Lüthje's figures have been recalculated by us as percentages of the final body-weight of the animals and as fat-free body-weight; and are shown in Table I.

Table I. *Percentage content of solids, fat and nitrogen in two castrated dogs as compared with that of normal litter mates.*

	Final weight of dog in g.	Solids %	Fat %	Nitrogen %	Nitrogen per 100 g. fat-free body-weight
Normal ♂	17,650	46·5	9·2	3·01	3·31
Castrated ♂	16,600	47·5	8·6	3·14	3·44
Normal ♀	11,400	45·3	9·15	2·93	3·22
Castrated ♀	12,620	48·1	12·8	2·81	3·23

As was stated in our experiments on dogs [Korenchevsky, 1925], only some of these animals became obese after castration. Similarly in Lüthje's experiments one dog (male) became a "thin" castrate after castration, whilst the bitch was a typical "fat" castrate. Consequently, the results of chemical investigation were different, the general nitrogen content being slightly greater (by 4·3 %) in the "thin" castrate, whilst in the castrated bitch it was slightly less (by 4·1 %), than in the respective control animals. This decrease of nitrogen content in the obese bitch was not evident when the nitrogen content was recalculated per 100 g. of fat-free body-weight. This decrease of nitrogen in the "fat" castrate is therefore only apparent and due to the increased proportion of fat in the body and not to an impoverishment in protein substances of the castrated organism. There was, moreover, a slight increase in the nitrogen of the "thin" castrated male dog. It was shown in a previous paper [Korenchevsky, 1925] that, after castration, the deposition of nitrogen in the body of most castrated animals increases. This increase, indicated by metabolism experiments, agrees well with an increased nitrogen content of the body of the "thin" castrate found in Lüthje's experiment (Table I). An increase in the solids in the bodies of both castrated dogs (see Table I) can be explained by the deposition either of fat, or of nitrogen, or of both.

PRESENT EXPERIMENTS.

The experiments were performed on 46 rats, belonging to 9 litters (see Table II). Each litter was divided into a group of control rats and groups of cryptorchid and castrated rats. The cryptorchids of some litters were divided into one-sided cryptorchids, in which only one testicle was made cryptorchid, and into both-sided cryptorchids in which both testicles were made cryptorchid. Altogether 18 control rats, 5 one-sided cryptorchids, 16 both-sided cryptorchids and 7 castrates were used. All these rats have been described elsewhere [Korenchevsky, 1930] and all the details of the general technique employed are there given, as well as a description of the influence of cryptorchidism and castration on growth, fat deposition, sexual and endocrine organs. In the present investigation, it was decided to use the hind legs for analysis, since they are suitable as a general indication of the chemical composition of the principal tissues of the body, namely, skin (with fur), muscles and skeleton. Two controlling chemical analyses are obtainable, so that the results obtained on one

Table II. *The percentage content of nitrogen in each dry hind leg of each rat.*

	Normals		Cryptorchids: one-sided		Cryptorchids: both-sided		Castrates	
No. of litter	No. of rat	Nitrogen content	No. of rat	Nitrogen content	No. of rat	Nitrogen content	No. of rat	Nitrogen content
5	829	10·21	835	9·88	831	9·58	834	8·92
		10·25		10·48		9·63		8·88
	830	9·90			832	10·24		
		9·89				10·14		
Average		**10·06**		**10·18**		**9·90**		**8·90**
6	836	9·80	838	9·32	839	9·28		
		9·75		8·84		8·96		
	837	9·61			840	—		
		9·58						
	841	9·83						
		9·69						
Average		**9·71**		**9·08**		**9·12**		
7	842	—	843	10·01	844	9·50		
				10·26		9·53		
	845	10·09			846	10·17		
		10·11				10·04		
Average		**10·10**		**10·14**		**9·81**		
8	863	9·60	865	9·45	866	9·07	868	9·20
		9·65		9·73		8·91		9·30
	864	10·01						
		9·86					867	8·77
								8·39
Average		**9·78**		**9·59**		**8·99**		**8·92**
9	869	9·04	872	9·99	871	9·55	873	8·94
		9·22		9·65		9·47		8·82
	870	9·57					874	9·42
		9·74						9·62
Average		**9·39**		**9·82**		**9·51**		**9·20**
22	847	9·78			849	9·35	851	9·12
		9·75				9·29		8·94
	848	9·49					852	9·59
		9·43						9·63
Average		**9·61**				**9·32**		**9·32**
23	856	9·85			854	10·10		
		9·87				9·87		
					855	9·84		
						9·91		
					857	9·73		
						9·70		
Average		**9·86**				**9·86**		
24	858	—			861	9·73		
						9·75		
	859	11·23			862	9·62		
		10·54				9·87		
Average		**10·89**				**9·74**		
25	875	9·68			880	9·28		
		9·53						
	877	10·04			881	10·13		
		10·11				10·18		
Average		**9·84**				**9·86**		
Average of averages of litters		**9·92**		**9·76**		**9·57**		**9.09**

leg can be used to check the results obtained with the other. It was decided to investigate the amount of water, solids and nitrogen in the legs, but it is obvious that any other necessary chemical analyses could also be made. For the approximate estimation of the deposition of fat in the body, a still simpler method, which was used by us in the previous paper [Korenchevsky, 1930], was chosen. In this method, the weight of the retroperineal and testicular fat, which can be quickly and easily separated from the body by means of a scalpel, was taken for the comparative estimation of the degree of deposition of fat in the animal. The comparative changes in fat deposition in different animals can be checked in each animal by the separate weighing of the testicular and retroperineal fats. Elsewhere [Korenchevsky, 1930], only the total amount of intra-abdominal fat (*i.e.* the retroperineal plus testicular) is given. Therefore in the present paper we give, in Table III, the weights of retroperineal fat and of testicular fat separately, so that both data can be compared. The weight of fat is calculated in the table as g. per 200 g. body-weight of the rat. The separation of the legs from the body was performed in the following way: the hind leg was bent at the fibula-tibia-femoral junction by the left hand of the investigator and the blades of the scissors placed round and just a little above this junction, in such a manner that the lower blade of the scissors was placed in the angle made by bending the leg at the junction; then the leg was cut off, without producing tension of the skin and muscles and the cut made neat with the scissors. It was possible, without any special training, to sever the legs so accurately, that the dry weight of the right leg of the animal differed from that of the left leg of the same animal, on the average, by only ± 2·6 % (minimum ± 0·2, maximum ± 9·1 %). The legs were placed in weighing bottles and weighed, first fresh and then after desiccation at about 103°. The nitrogen was estimated by the Kjeldahl method in each leg separately. In order to save space only one table, No. II, is given in detail. In all the other tables the changes obtained in groups of litters, and not those in individual rats, are given.

Table III. *Average weight of retroperineal and testicular fat in groups of normal, cryptorchid and castrated rats.*

	Normal rats		One-sided cryptorchids		Both-sided cryptorchids		Castrates.
No. of litter	Retro-perineal fat	Testi-cular fat	Retro-perineal fat	Testi-cular fat	Retro-perineal fat	Testi-cular fat	Retro-perineal fat
5	6·27	5·70	8·60	8·24	4·13	6·68	12·91
6	6·41	6·97	8·59	9·77	10·02	7·98	—
7	6·96	6·54	6·81	8·49	5·22	9·21	—
8	6·47	8·40	8·28	9·97	8·07	7·95	11·41
9	8·68	7·18	6·02	6·78	8·89	9·91	9·36
22	6·97	5·98	—	—	8·15	9·96	9·67
23	5·27	5·79	—	—	7·35	4·98	—
24	4·32	5·35	—	—	7·74	8·48	—
25	5·77	4·94	—	—	6·66	8·95	—
Average	6·35	6·32	7·66	8·65	7·36	8·23	10·84

FAT DEPOSITION.

From a comparison of the weights of retroperineal and testicular fat in Table III, it is possible to say that in five of the groups of cryptorchid rats, of litters nos. 5, 7, 8 and 23, there is a lack of agreement in the changes obtained between testicular and retroperineal fats, namely that, whilst the testicular fat, as compared with that of the normal control rats, was increased, the retroperineal fat was decreased or showed no change, or *vice versa*. This indicates that, whilst it is only in the majority of cases that the increase, or decrease, in fat deposition is evenly distributed between the retroperineal and testicular regions, in some rats fat is deposited in one or other of these regions preferentially. It is therefore necessary, in animals with normal testes, to use the sum of the weights of the testicular and retroperineal fats to obtain an indication of the deposition of fat in the body. However, when a sufficiently large number of animals is used for the conclusions, the average figures give similar results in both cases. A comparison of the average figures of Table III shows that, on the average, the weights of both the retroperineal and testicular fats were increased in cryptorchid animals, as compared with those of normal control animals. The amount of fat in castrated animals was also increased and in all cases to a greater degree than in cryptorchid animals. For further conclusions based on the total intra-abdominal fat and on a larger number of animals we refer to the previous paper [Korenchevsky, 1930].

NITROGEN CONTENT.

In Table II is given the percentage content of nitrogen in each dry hind leg of each rat. The average for each group of each litter and a general average for all the rats of the group are also given. It can be seen, by comparing the nitrogen content of the two legs of the same rat, that in the 42 animals investigated, the fluctuations, from the average for the two legs of the same rat, was about or less than ± 0·5 % in 21 rats, from ± 0·5 to ± 1 % in 10 rats, from ± 1·1 % to ± 1·75 % in 7 rats and ± 2 to ± 3·2 % in 4 rats.

These results indicate that the method employed was quite satisfactory, giving data sufficiently accurate for the comparison of the different animals, with a probable error not greater than 1·8 % in most cases and in no case investigated more than 3·2 %.

On a general average, it was found that the legs of one-sided cryptorchids contained 1·6 %, of both-sided cryptorchids 3·5 % and of castrates 8·4 % less nitrogen than the legs of their normal controls. Consequently it is necessary to note that the castrated rats were more obese than the cryptorchids and both of them were more obese than the normal rats. We consider therefore that we are justified in explaining this small decrease in the nitrogen content in the same way as we did for Lüthje's dogs (p. 955); that it is unlikely that it can indicate a decrease in the amount of nitrogenous substances in the bodies of cryptorchid and castrated rats; that the decrease is apparent and is due to the increased proportion of fat in the cryptorchid and castrated animals. This is in agreement with the cases where the nitrogen content was even slightly increased, as was the case in the one-sided cryptorchids of groups 5, 7 and 9 and in the both-sided cryptorchids of group 9. In all these groups the amount of solids (Table IV) was less, in spite of the increased amount of fat in most of the groups. Therefore an even higher nitrogen content is suggested in these cases.

Table IV. *The average percentage content of solids and water in groups of normal, cryptorchid and castrated rats.*

No. of litter	Normal rats		One-sided cryptorchids		Both-sided cryptorchids		Castrates	
	Solids	Water	Solids	Water	Solids	Water	Solids	Water
5	38·1	62·0	37·4	62·6	38·9	61·1	40·8	59·2
6	39·1	60·9	40·2	59·8	41·4	58·7	—	—
7	38·9	61·1	37·7	62·3	39·6	60·4	—	—
8	39·4	60·6	39·7	60·3	41·7	58·3	41·8	58·2
9	40·6	59·5	39·8	60·2	40·0	60·0	40·2	59·8
22	39·6	60·5	—	—	40·4	59·6	39·8	60·2
23	39·1	60·9	—	—	39·7	60·3	—	—
24	37·1	63·0	—	—	40·1	59·9	—	—
25	39·2	60·8	—	—	40·1	59·9	—	—
Average	39·0	61·0	39·0	61·0	40·2	59·8	40·7	59·4

SOLIDS AND WATER CONTENT.

From Table IV it is clear that the solids content of the legs was slightly higher, and the water content slightly less, in both-sided cryptorchids and in castrates, than in the normal control rats. On the average, there was no change in one-sided cryptorchids.

Although this increase in the content of solids was only small (by 3·1 % in cryptorchids and 4·4 % in castrates) nevertheless its occurrence in nearly all the groups investigated indicates that it is not of an accidental character. The increased deposition of fat is probably only partly responsible for this increase, since in one-sided cryptorchids the degree of obesity was about the same as in both-sided cryptorchids, but the increase in solids was absent in the former group.

Taking into consideration the increased deposition of nitrogen in most castrated animals [Korenchevsky, 1925], we suggest the same explanation as we have given for Lüthje's results, namely, that in castrated and both-sided cryptorchid animals an increase in the content of solids is to be explained by an increased deposition of fat and probably also of nitrogenous substances in the body.

SUMMARY.

1. A method was used for the investigation of the chemical composition of small animals, which gives approximate but accurate data.

2. This method was applied for ascertaining the content of water, solids, nitrogen and fat in castrated and one-sided and both-sided cryptorchid male

rats, as compared with that of normal litter mate animals. Altogether 46 rats were investigated.

3. The amount of intra-abdominal fat was increased in both cryptorchid and castrated animals, but to a greater degree in the latter.

4. The nitrogen content was slightly decreased in both-sided cryptorchid and in castrated rats.

5. This decrease, however, is chiefly due to the increased proportion of fat in the bodies of cryptorchid and castrated rats, and therefore is not due to the impoverishment of the bodies of these animals in nitrogenous substances.

6. A slight increase in the content of solid matter was found in both-sided cryptorchid and in castrated rats. This increase can be explained by an increase in the deposition of fat and probably also of nitrogenous substances.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled us to carry out this work and to them our thanks are due. We also wish to express our sincere gratitude to Sir Charles Martin for his continuous support and criticism of this work.

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CVII. AN IMPROVED DESIGN OF THE VAN SLYKE APPARATUS FOR THE ESTIMATION OF AMINO-NITROGEN.

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(*Received June 28th, 1930.*)

An extended use of the apparatus described by Van Slyke [1912, 1914, 1915] for the quantitative determination of amino-nitrogen has revealed several drawbacks.

There are three points in the glass system at which pieces of thick-walled glass capillary tubing are joined by rubber tubing. The undesirability of rubber connections in systems in which gas volumes are measured needs no emphasis. In the case of the Van Slyke apparatus the abolition of the rubber connections is the more desirable in that shaking of the deaminising chamber and Hempel pipette subjects the rubber to periodic stretchings which must diminish its impermeability to gases. Where deamination takes 1 hour, as it does in the case of lysine, histidine, etc. [Plimmer, 1924], this disadvantage may be serious. It was found necessary always to apply glycerol to the surfaces of contact of glass and rubber and tightly to wire the rubber to the glass.

If thick-walled rubber tubing is used in order to lessen the danger of loss of gas at the joints, shaking subjects the thick-walled glass capillary tubing to strain, on account of the elasticity of the rubber. In use, breaks were frequently found to occur.

These considerations prompted the design of a new form of the apparatus in which the disadvantages referred to were eliminated.

The gas system is made in one piece and mounted without strains on a stout, upright board, having apertures cut in it where necessary. Fig. 1 shows a front view of the apparatus. *A* represents an end elevation of the deaminising bulb which is of the original Van Slyke design. The deaminising bulb is clipped to a wooden block, which is screwed to the board and is not indicated in the figure. The thick-walled capillary tubing as well as the two-way tap *B* are of larger size than usual in order to impart greater strength to the apparatus.

The gas burette is of 10 cc. capacity, graduated in its upper part in 0·01 cc., the bore being as large as conveniently possible. The bulb at its lower end holds 40 cc. A bulb of this size was found necessary when hexone bases were analysed, since at the end of the hour necessary for deamination considerably more than 20 cc. of gases have been evolved.

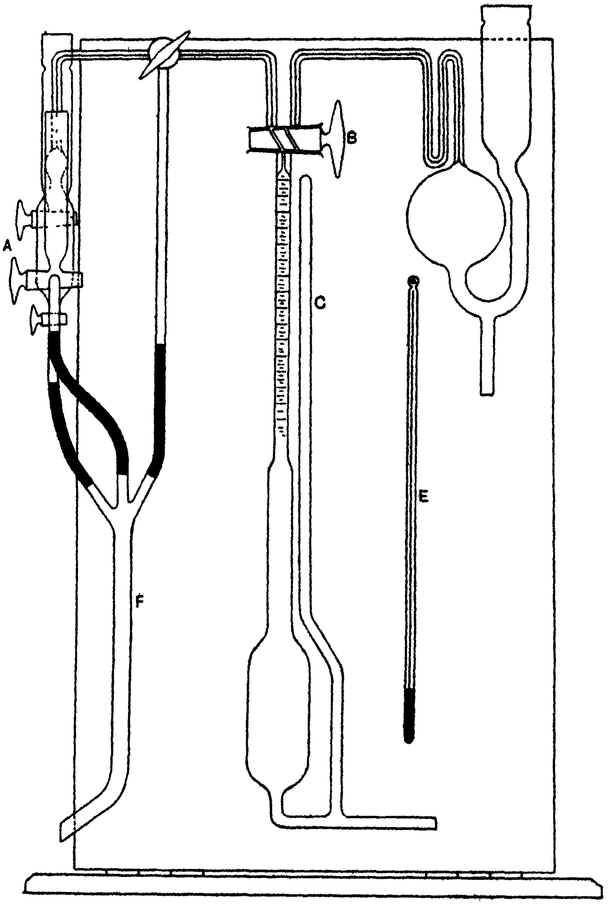


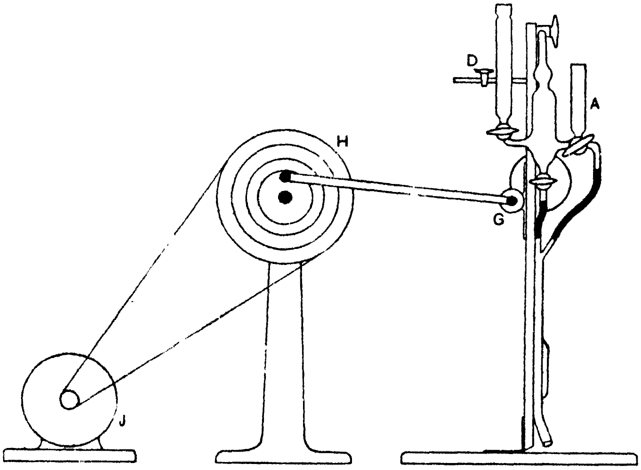
Fig. 1.

The levelling manometer *C* provides greater ease and accuracy in adjusting gas volumes to atmospheric pressure. It is bent over at right angles at the top and fitted with a tap *D*, shown in Fig. 2, so that the manometer can be closed when the manipulations necessitate it. Its diameter should be large enough for the capillary rise of water to be negligible.

The absorption pipette has been slightly modified from the original, the spherical bulb being of 100 cc. capacity, while the cylindrical vessel holds 60 cc.

A thermometer *E* and waste pipe *F* are also fastened to the board, the latter communicating by rubber connections with the three outlets of the deaminising bulb.

The upright board is fastened by strong hinges at the bottom to a base-board and can be oscillated about the hinges by means of a crank attached to the board by the brass coupling *G*. The crank is actuated by the wheel *H* driven by an electric motor *J*, a considerable variation in the speed of shaking being afforded by a suitable rheostat. The whole apparatus is firmly screwed to the bench.



The levelling reservoir is placed in a cradle which can be slowly raised or lowered by means of an endless screw.

With this apparatus it was found possible to estimate amino-nitrogen to 0·01 mg. A standard solution of twice crystallised copper aminoacetate was prepared and the quantity of amino-nitrogen in 2 cc. determined, the solution being run in from a calibrated pipette and washed in with 2 cc. water.

Barometer mm. mercury	Temp.	cc. nitrogen evolved	mg. amino-nitrogen
765	18·8°	1·98	1·14 (1)
765	19·0	1·99	1·14 (6)
767	20·0	1·96	1·12 (7)
767	21·0	2·00	1·14 (4)

Theoretical value 1·13 mg.

In 1915, Van Slyke described a modification of his original apparatus in which, it was claimed, volumes of nitrogen could be measured to 0·001 cc. by means of a burette graduated in 0·01 cc. with the graduations about 1 mm. apart.

It seems doubtful whether this degree of accuracy can generally be attained without special precautions to ensure the constancy of the temperature of the gas burette and also to ensure that there is no temperature lag between the latter and the thermometer. A temperature variation of 0·5° will make an appreciable difference in the third decimal place of milligrams of nitrogen.

Further, it seems inadvisable to use a gas burette of so narrow a bore as that specified by Van Slyke, since in such a tube the capillary rise of water will be appreciable. Hence the gaseous volumes will be read under a slightly different pressure from that recorded by the barometer.

During the manipulations it is almost impossible to avoid contamination of the water in the upper part of the burette with acetic acid, sodium nitrite, potassium permanganate and sodium hydroxide, and it is certain that such capillary active substances will appreciably affect the surface tension of the liquid over which the gas is measured and hence change the capillary rise. The pressure of water vapour in the burette will also be changed by the presence of these impurities.

Though the magnitude of these effects is not accurately known, consideration of them makes it evident that, until they have been shown to be negligible, volumes of nitrogen under these conditions can only be given with certainty to 0·01 cc.

I should like to acknowledge the facilities afforded by the Physiology Department of the University of Manchester and to express my thanks to Prof. H. S. Raper and Mr A. D. Ritchie for kind advice.

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CVIII. A SENSITIVE REACTION FOR CYSTEINE.

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Sullivan [1926] found that cystine reduced with sodium cyanide and sodium sulphite gives a colour with sodium β-naphthoquinone-4-sulphonate. This substance is used by Folin [1922] for the estimation of amino-acids and so would appear not to be specific for cysteine. According to Hunter and Eagles [1927], however, the colour given by other amino-acids is not the same as that given by cysteine. The importance of cysteine in biological chemistry and the labour involved in the preparation of the naphthoquinone reagent led us to investigate the possibility of finding a more simple and specific test. We found that cysteine hydrochloride reacts readily with dimethyl-*p*-phenylenediamine hydrochloride when heated in presence of a small quantity of $FeCl_3$ giving a deep blue colour. The colour is stable. It resembles that of methylene blue but does not match it exactly. Like the latter it can be precipitated in presence of $ZnCl_2$ and NaCl at high concentrations. The cysteine employed by us was supplied by the British Drug Houses. We also used other samples prepared from a sample of Merck's cystine by reduction with zinc in hydrochloric acid solution. The solution of cysteine hydrochloride was made by dissolving 0·04 g. in 100 cc. of water, and the solution of dimethyl-*p*-phenylenediamine hydrochloride by dissolving 0·2 g. in 100 cc. of water. It is preferable though not absolutely necessary to use a freshly prepared solution of the latter, as on standing for some time it develops a purple colour. If, however, the purple colour is not too pronounced the addition of the cysteine solution usually renders it colourless again. The concentration of the $FeCl_3$ solution was 5 %. In testing for very small quantities of cysteine, however, it is preferable to use a more dilute solution of $FeCl_3$. The most suitable proportions in which the reagents should be mixed are as follows: to 0·5 cc. of the *p*-phenylenediamine reagent should be added 1 cc. of the cysteine solution followed by one drop of the $FeCl_3$ solution. On heating the mixture almost to boiling point and allowing it to stand the deep blue colour is produced. The mixture of cysteine and *p*-phenylenediamine reagent may first be heated together and the drop of $FeCl_3$ then added to the hot solution when the blue colour develops as before. The test is sensitive with 0·05 mg. of cysteine hydrochloride.

Under similar conditions soluble sulphides do not give methylene blue but require a much more acid medium. The methylene blue reaction for sulphides is given in the cold.

Cystine under the conditions above mentioned gives no colour with these reagents but of course reacts on reduction. Thiolactic and thioglycollic acids do not react with these reagents. Using yeast extracts as a source of glutathione we found that neither oxidised nor reduced glutathione reacted with our reagents. On hydrolysis, extracts of fresh yeast give a positive reaction indicating the presence of cysteine in the decomposition products of glutathione. We hope soon to be able to publish a method, based on this reaction, for the estimation of cysteine and glutathione in tissue extracts.

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CIX. VITAMIN C AND THE RAT'S DIET.

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The rôle of the antiscorbutic vitamin in the rat's diet is a question upon which there appear to be different views. On the whole nutrition workers in America omit orange and lemon juice from synthetic diets, while in England it is usual to add small quantities. Simmonds [1924] states that "the rat is apparently immune to scurvy and does not require this vitamin in its diet," and Mendel [1923], in discussing the essential factors of a diet, says that the rat does not seem to suffer from a lack of antiscorbutic foods. Parsons [1920] found that the livers of rats fed for long periods on a typical scorbutic diet were rich in the antiscorbutic substance and suggests that the rat needs this factor for its normal metabolism, but is capable of synthesising it. Many American workers do not even mention vitamin C in discussing the composition of their rats' diets, but tacitly assume that this food factor is not needed by the animal. For instance, Anderson and Mendel [1928] supplement their experimental diets by daily doses of yeast and cod-liver oil to provide "an adequate intake of the indispensable vitamins." Sure [1924, 1925] uses cod-liver oil and Harris uses yeast-vitamin powder, but no orange or lemon juice either with his skimmed milk or pure synthetic diets, and Elmslie and Steenbock [1929] give yeast as a source of the vitamin B complex and cod-liver oil to supply vitamins A and D, but no vitamin C. Kennedy and Palmer [1922] also use no vitamin C in their basal diet and express the view that very few workers regard vitamin C as a necessary constituent in the diet of growing rats. Evans and Bishop [1923] used no source of vitamin C in their synthetic diet, but in trying to cure sterility they gave orange juice to some rats and fresh green lettuce to others; they found the latter to be a most efficient cure, but apparently noted no effects due to addition of the orange juice. On the other hand, Harden and Zilva [1918] consider that rats grow better when the antiscorbutic factor is present in the diet, and their growth curves exhibit an appreciable improvement. Drummond [1919] agrees with the work of Harden and Zilva and states that "the rat requires the antiscorbutic factor in order to achieve a normal development." Kennedy [1926] says that although the rat appears to thrive without vitamin C he added concentrated orange juice in case it might be essential for reproduction. The experiments of Bacharach [1925] showed that no disadvantage attended the omission of lemon juice from the rat's diet during the experimental period involved in testing for vitamin B, and other workers [Coward, Key, Morgan and Cambden, 1929; Drummond and Marrian, 1926; Reader, 1929; Willimot, 1926] now omit lemon juice from a vitamin B-free diet. Coward [1929, 1930] and Coward, Key and Morgan [1929] also omit vitamin C from their vitamin A-free diet.

The amount of orange or lemon juice added to synthetic diets varies a little, but as a rule 5–6 cc. per 90–100 g. dry solid is given by nutrition workers in England [Boas, 1926; Chick, 1926; Chick and Roscoe, 1926; Luce and MacLean, 1925; Reader and Drummond, 1926].

The diets used by Harden and Zilva [1918] contained much larger amounts of lemon juice, *i.e.* 40 instead of 5 cc. Since the growth of rats fed on synthetic diets is far below that obtained on a good mixed diet of kitchen scraps, it is worth while to try any factor which might produce an improvement. Therefore experiments were made in which rats were given large amounts of lemon juice in the synthetic diet to see if it would be an advantage to incorporate more than the usual 5 cc. of orange or lemon juice in the basal diet.

Experimental.

(*a*) *Animals and method.*

Piebald rats bred in the laboratory were used in this work. Twelve animals, 6 ♀ and 6 ♂ (except in the trial experiment with lemon juice, where owing to a shortage of rats only 5 ♀ and 4 ♂ were available) were used for each experiment and were kept in a large metal cage with a screen, so that they had no access to excreta. The pregnant does were removed to smaller wooden cages a few days before the birth of a litter. The rats were weighed daily for the first 4 weeks, after which the weighing was omitted on Saturdays and Sundays. After the birth of the first litter, when the rate of growth is slower, the males were weighed only once a week, though the mother and her young were weighed daily.

(*b*) *Diets.*

Two basal diets were used, one containing caseinogen and the other edestin, as it was thought advisable to use two different proteins in case one produced greater growth than the other, as a result of which the effect of vitamin C might be more marked. The composition of the basal diets was as follows:

Diet AF	Diet AF Ed
Caseinogen 20 g.	Edestin 20 g.
Potato starch 56 g.	Potato starch 56 g.
Mineral salts 8 g.	Mineral salts 8 g. [Hartwell, 1922].
Butter 16 g.	Butter 16 g.
Marmite 15 g.	Marmite 15 g.

The diet was prepared by the "cooked" method described in a previous paper [Hartwell, 1925]. Lemon juice strained through muslin and the green leaves of cabbage finely minced were used as sources of vitamin C, and both were well beaten into the diet when it had cooled. Food and distilled water were given *ad lib.*, but special feeding dishes were used in order to prevent scattering of the food and thus to obtain an approximate intake per cage.

(*c*) *Experiments.*

1. *Diet AF.* This experiment was made in duplicate in order to see what differences in growth might occur with the same diet.

2. *Diet AF + lemon juice.* The proportion of lemon juice added was 40 cc. to every 84 g. of dry diet (*i.e.* caseinogen, starch and mineral salts). A trial experiment was made using 5 ♀ and 4 ♂ to see if the animals could tolerate this amount of lemon juice, or if such a diet would be too acid and therefore cause intestinal disturbances. The young rats developed diarrhoea after 3–4 days on the diet, but after 10 days the faeces were quite normal. Temporary digestive disturbances often occur when weanling rats are put on synthetic diets, but usually the young animals appear to be able to adjust themselves to their new diet in a week or 10 days. In a second experiment 6 ♀ and 6 ♂ were used, and the experiment was continued for 15 weeks.

3. *Diet AF + cabbage.* 72 g. minced green cabbage leaves were added to the basal diet instead of 40 cc. of lemon juice. For the first 2 weeks minced lettuce was given, but this proved so expensive that green cabbage was used instead.

4. *Diet AF Ed.* This diet was similar to diet AF, but pure edestin[1] was used instead of caseinogen.

5. *Diet AF Ed + lemon juice.* 40 cc. lemon juice (strained) were added to 84 g. of the dry diet as in the experiment AF + lemon juice.

RESULTS AND DISCUSSION.

(1) *Growth.* In all experiments the rats grew well, though the rate of growth was not equal to that of the control animals fed on a mixed diet of kitchen scraps + bread and milk. The addition of lemon juice appeared to have no appreciable effect on the rate of growth. Fig. 1 shows the average growth curves of the males and of the females up to the time they became pregnant. The curves show that there is no greater difference in weight between the rats receiving lemon juice and those getting no vitamin C than there is between the two curves obtained at different times on diet AF alone. In the trial experiment with lemon juice the growth is just better than when no lemon juice was included in the diet. The growth curves when cabbage was used as a source of vitamin C show similar results. When edestin was given

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[1] I am indebted to Messrs E. T. Pearson & Co., Ltd., for the pure edestin used in these experiments.

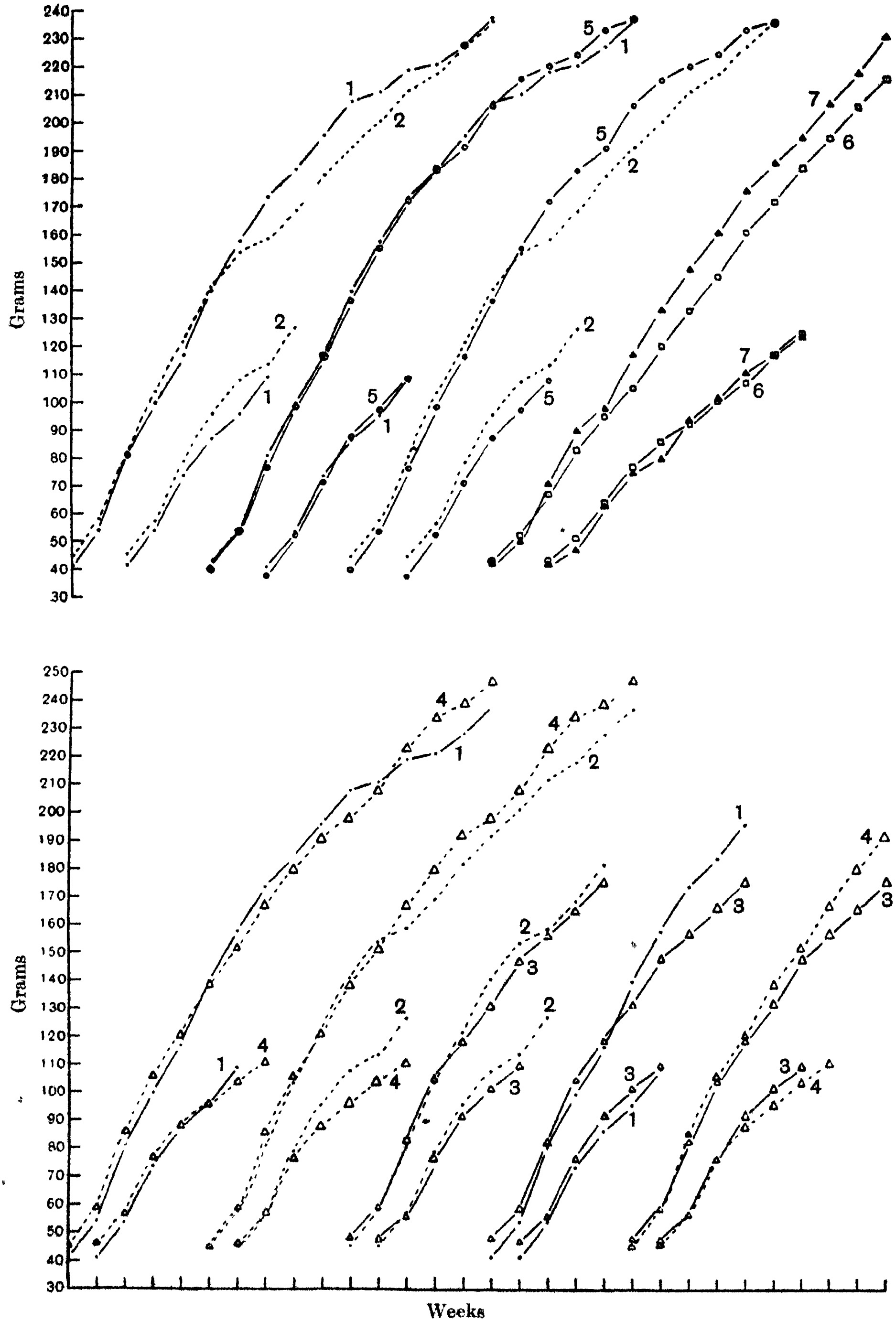

Fig. 1. Growth curves of male and female (shorter owing to pregnancy) rats. Each curve is the average for six rats, except diet AF + lemon juice, Exp. 1.

1. • ——— • Diet AF. Exp. 1.
2. • • • • • • • Diet AF. Exp. 2.
3. △——△ Diet AF + lemon juice. Exp. 1.
4. △- - -△ Diet AF + lemon juice. Exp. 2.
5. ○——○ Diet AF + cabbage.
6. □——□ Diet AF Ed.
7. ▲——▲ Diet AF Ed + lemon juice.

instead of caseinogen the rate of growth appeared to be just better when lemon juice was added, but this improvement is very slight, the actual difference in weight being no greater than that obtained in the two separate experiments on diet AF.

(2) *General condition.* Although the animals grew well, their general condition was not up to standard. None of them was quite so firm to feel as the stock animals and practically every rat exhibited loss of fur, though the coat grew again without any alteration in the diet. The lemon juice caused no improvement in the caseinogen diet, but with edestin the fur did not come out quite so badly when lemon juice was added.

(3) *Reproduction.* On the whole reproduction was very poor and therefore the experiments were not continued after the first litters were born. Both with presence and absence of lemon juice the young were frequently born dead or died within a few days of birth, only a few were reared and the numbers per litter were low compared with stock animals.

From these experiments, therefore, it appears that the addition of lemon juice to the basal diet produces no improvement in breeding, but green cabbage leaves have a beneficial effect. Hence it seems that vitamin C itself is not the factor to which the improvement is due, and it is obvious that the raw cabbage contained some food factor of importance in breeding, which was not present in the lemon juice. It seems unlikely that the diets used were at all deficient in vitamin E, for Sure [1927] finds that 10 % of butter-fat in a synthetic ration assures fertility and successful lactation in the rat, and the diets described in this paper contained 16 % of butter. Mattill and Clayton [1926] consider it probable that milk-fat contains more vitamin E than has been estimated, though Evans and Bishop [1923] find that large quantities of this fat are required to cure sterility. However, the curative dose may be greater than the preventive ration. No information can be found as to the relative amounts of vitamin E in lemon juice and cabbage, therefore the reason for the improvement produced by cabbage leaves must await further research. It is interesting, however, to note that no improvement in growth was obtained by the addition of cabbage as compared with the lemon juice, affording further evidence that the dietary requirements vary with different periods of existence.

The experiments described here do not support the view of Harden and Zilva [1918] that the addition of the antiscorbutic vitamin to the rat's diet produces better growth. These workers obtained distinctly better growth when they added lemon juice with the citric acid removed, but as suggested by Palmer and Kennedy [1927] the lemon juice may have contained vitamin A or B, or both, and therefore the favourable result may not have been exclusively due to vitamin C. Since the experiments of Harden and Zilva much work has been done on growth with various diets and it is probable that there are several possible explanations of their results. For instance, their AB diet contained different amounts of the various constituents as compared with their ABC diet—thus in the AB diet there were 2·5 cc. olive oil, while the ABC diet had only 0·25 cc.

More recent work has shown that the actual proportion of constituents of a diet is an important factor and this in itself may account for the different rates of growth. It is also possible that the modified technique adopted now, *i.e.* keeping animals on screens away from their excreta, will require an alteration in the amounts of the different food factors required. In previous work [Hartwell, 1926] good growth was obtained with 2 g. marmite per 100 g. solid, but more recent work when rats were kept on screens has shown this figure to be too low. With their excreta accessible animals make up deficiencies in their diets from this source, but a more generous supply in their food may well produce better growth.

SUMMARY.

The addition of the antiscorbutic vitamin to a synthetic diet produced no improvement in the rate of growth or reproduction of the rat.

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CX. STUDIES ON HEMICELLULOSES.

II. THE HEMICELLULOSES OF MAIZE COBS.

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INTRODUCTION.

MAIZE cobs have long been recognised as a source of xylose, which may be obtained from them in a pure condition and in fairly high yield. More recently, a method for the preparation of xylose from this source has been described by Ling and Nanji [1923], who obtained the sugar in 10–12 % yield.

This xylose arises, at least in part, by the hydrolysis of hemicelluloses present in the cobs. Johnson [1896] investigated the nature of maize cob hemicellulose. After a preliminary treatment with dilute ammonia, the cobs were extracted with sodium hydroxide solution, the hemicellulose being precipitated by addition of alcohol. The product yielded xylose on hydrolysis, other sugars not being detected, while as a result of elementary analysis the formula $(C_5H_8O_4)_n$ was ascribed to it.

Maize cob hemicellulose was thus regarded as a single substance, indeed as a pure xylan.

However, it has been indicated in a previous paper dealing with the hemicelluloses of wheat bran [Norris and Preece, 1930] that in some cases at least it may be possible to separate the hemicellulose from a given source into a number of fractions, having constitutions which are similar but, nevertheless, distinct. Moreover, it has been shown that uronic acid residues are widespread in hemicelluloses [O'Dwyer, 1926; Norman, 1929].

It was felt, therefore, that the method of separation successfully applied in the case of the hemicelluloses of wheat bran might to advantage be attempted with the hemicelluloses of maize cobs, with the added object of detecting the presence of constituents other than xylan. In a substance of such complexity as a hemicellulose, the substitution of one or more residues of xylose by residues of other sugars, or sugar derivatives, would but slightly alter its elementary composition. Thus, the analysis of maize cob hemicellulose might still approximate very closely to that for a pure xylan, even though xylose were not the only sugar obtainable from it on hydrolysis.

EXPERIMENTAL.

Preparation and purification of the hemicelluloses.

As a preliminary to chemical treatment, the cobs were mechanically reduced to a coarse sawdust. The preparation of the hemicelluloses was carried out in exactly the same way as has been described for the preparation of the hemicelluloses of wheat bran [Norris and Preece, 1930]. The method there described again gave three fractions, designated hemicelluloses A, B and C.

Furfuraldehyde determinations were carried out on the products, and the results obtained (calculated on the dry, ash-free material) are quoted in Table I where the yields from 400 g. of the ground cobs are also given.

Table I.

Fraction	Yield of crude product from 400 g. of cobs (g.)	Furfuraldehyde %
A	32	60·70
B	24	57·82
C	6	50·77

Hemicellulose A was purified by dissolving in 4 % sodium hydroxide solution, filtering through paper pulp, and precipitating with Fehling's solution as the copper complex. This was decomposed with dilute hydrochloric acid, and the hemicellulose precipitated and washed with acetone.

An attempt was made to separate hemicellulose B into two fractions analogous to hemicelluloses B 1 and B 2 of wheat bran. However, the whole of this fraction was directly precipitated from alkaline solution by Fehling's solution; consequently this product is described as hemicellulose B 1.

On the other hand, hemicellulose C was split up by copper treatment, a portion—hemicellulose C 1—being directly precipitated by Fehling's solution, while hemicellulose C 2 was precipitated from the filtrate from this on addition of half a volume of acetone. Both fractions were further purified by a repetition of the treatment.

Thus, the hemicelluloses of maize cobs, like those of wheat bran, may be separated into four fractions, the two sets having, however, somewhat different characteristics. Thus, while under the present system of nomenclature the wheat bran-hemicelluloses are designated A, B 1, B 2 and C 2, those of maize cobs are A, B 1, C 1 and C 2.

General properties of the hemicelluloses.

The four hemicelluloses are white, amorphous, non-reducing powders. Fraction A is insoluble in hot water; B 1 swells up in cold water, and is completely dissolved on boiling; C 1 is partially dissolved by cold water, complete solution being obtained on boiling; C 2 shows gum-like swelling in cold water; hot water dissolves it entirely. None of these hemicelluloses gives an iodine coloration.

During the estimation of furfuraldehyde, it was found that the phloroglucide precipitates obtained from hemicelluloses B, C 1 and C 2 contained appreciable amounts of a substance soluble in 95 % alcohol to a red-brown solution, indicating the presence of methylpentosan in these hemicelluloses. Consequently, determinations of methylpentosan in presence of pentosan were carried out according to the method of Tollens and Ellett [see Browne, 1912]. The percentages of methylpentosan found and the percentage yields of furfuraldehyde are given in Table II. Carbon dioxide numbers were also obtained by the method of Nanji, Paton, and Ling [1925]. The rotatory powers of A, B 1 and C 1 in 4 % sodium hydroxide solution were also determined.

All the results given in this table are calculated on the dry, ash-free hemicelluloses, and in each case the figures quoted are the mean for a number of closely agreeing determinations.

Table II.

Hemicellulose	A	B 1	C 1	C 2
Yield from cobs %	8·0	6·0	1·0	0·5
Methylpentosan %	—	7·01	7·42	8·91
Furfuraldehyde %	61·49	57·31	54·57	43·90
Carbon dioxide %	0·94	1·30	1·85	1·39
Uronic anhydride %	3·76	5·20	7·40	5·56
Rotatory power $[\alpha]_D^{20^\circ}$	− 96°	− 108°	− 90°	—

Since hemicellulose C, before separation into its constituents, was found to give a furfuraldehyde yield of 50·77 %, its components C 1 and C 2 are present in the ratio of two parts of the former to one of the latter.

Hydrolysis.

Hemicellulose A. Some 5 g. of the hemicellulose were hydrolysed by heating under reflux for 4 hours with 250 cc. of 1 % sulphuric acid. The sulphuric acid was then neutralised by the addition of the requisite volume of saturated barium hydroxide solution, and the precipitated barium sulphate filtered off. The filtrate was evaporated *in vacuo* just to dryness, and the residue extracted twice with 50 cc. portions of absolute alcohol. The alcoholic extracts after filtering were united, the alcohol removed by evaporation, and drying completed *in vacuo*. The residue insoluble in absolute alcohol was twice extracted with 50 cc. portions of boiling 80 % alcohol, the united extracts being taken to dryness as before. The amount of residue after 80 % alcohol extraction was extremely small, and was neglected.

The bulk of the hydrolysis product was found to have passed into the absolute alcohol. The sugar of this fraction gave $[\alpha]_D^{20^\circ} = +18^\circ$, and melted after recrystallisation at 139°.

From a portion of this sugar an osazone was prepared. The product was pure xylosazone, having the characteristic crystalline appearance and melting after recrystallisation at 160°. The presence of xylose was confirmed by the preparation of the characteristic cadmium xylonobromide.

From the sugar soluble in 80 % alcohol a mixture of osazones was obtained. One, insoluble in hot water, had a crystalline appearance similar to that observed in the case of insoluble phenylhydrazine derivatives obtained from the hydrolysis liquors of certain of the hemicelluloses of wheat bran [Norris and Preece, 1930]. After purification it melted sharply at 160°. In all probability, this product is a derivative of glycuronic acid. The second osazone was that of xylose, and the presence of this sugar was confirmed by the preparation of cadmium xylonobromide.

Sugars other than xylose could not be detected in either of the two fractions.

In a second hydrolysis, a weight equivalent to 1·549 g. of dry, ash-free hemicellulose A was treated with 1 % sulphuric acid in the usual way, the sulphuric acid neutralised with the requisite volume of barium hydroxide solution, and the barium sulphate removed by filtration. The precipitate was carefully washed with water, and the filtrate and washings combined. The liquid still remained acid to phenolphthalein, and a further amount of barium hydroxide was added to bring the reaction to the neutral point. After evaporation to dryness *in vacuo*, the sugar was extracted several times with small quantities of boiling absolute alcohol, and the extracts filtered hot, and combined.

From the combined extracts there was recovered 1·45 g. of xylose, giving for $c = 3{\cdot}362$ $[\alpha]_D^{20^\circ} = +19{\cdot}5^\circ$. It is shown below that hemicellulose A contains 94·36 % of xylan, so that the present amount of xylose obtained represents some 88 % of the theoretical yield.

Hemicellulose B 1. The neutral hydrolysis liquor from hemicellulose B 1 was taken to dryness *in vacuo* and extracted successively with absolute alcohol and with 80 % alcohol. The bulk of the sugar was again found to pass into the absolute alcohol.

The sugar soluble in absolute alcohol gave $[\alpha]_D^{20^\circ} = +18^\circ$. An osazone prepared from it proved to be xylosazone, melting after recrystallisation at 158°. Cadmium xylonobromide was also prepared.

The product soluble in 80 % alcohol showed $[\alpha]_D^{20^\circ} = +19^\circ$. This also was xylose, yielding xylosazone melting at 158°, and yielding cadmium xylonobromide.

Hydrolysis products other than xylose could not be detected in either fraction, nor was a phenylhydrazine derivative insoluble in hot water obtained from either.

A hydrolysis on quantitative lines was carried out in a manner similar to that employed in the case of the second hydrolysis of hemicellulose A described above.

1·72 g. of hemicellulose B 1 yielded 1·58 g. of xylose, showing for $c = 2{\cdot}31$ $[\alpha]_D^{20^\circ} = +19^\circ$, and melting at 140–141°. This represents a yield of xylose approximating to 92 % of the theoretical, since, as is shown below, hemicellulose B 1 contains 87·60 % of xylan.

Hemicelluloses C 1 and C 2. Owing to the small amounts of these products available for examination, only preliminary investigations of the nature of the sugars arising on hydrolysis could be attempted. However, these sufficed to indicate with certainty the nature of the pentoses produced.

To the hydrolysis liquor obtained from 1 g. of hemicellulose C 1 saturated barium hydroxide solution was added until the liquid remained just acid to phenolphthalein. The filtrate from barium sulphate was concentrated to a bulk of 50 cc. and divided into several portions for examination.

An osazone was prepared. The product was mainly pentosazone, soluble in hot water. This had the crystalline form of xylosazone and melted after several recrystallisations at 154°, a somewhat low temperature. The small amount of derivative insoluble in hot water had the crystalline form associated with the phenylhydrazine derivative of glycuronic acid. It was not further examined.

The presence of xylose was confirmed by the preparation from a second portion of hydrolysis liquor of the characteristic cadmium xylonobromide. Arabinose and hexoses were absent.

The hydrolysis liquor from 1 g. of C 2 was treated similarly to the above. The osazone obtained was again a mixture. The small amount insoluble in hot water was the glycuronic acid derivative, melting after recrystallisation from dilute alcohol at 155–156°. The hot water-soluble osazone resembled in crystalline appearance the osazone of arabinose; it melted at 158°.

By treating a portion of the hydrolysis liquor in the usual way with diphenylhydrazine, a copious precipitate of colourless crystalline arabinose diphenylhydrazone was obtained on standing. After recrystallisation from 50 % alcohol this gave a melting point of 200°. The presence of arabinose amongst the products of hydrolysis of hemicellulose C 2 was thus confirmed. Xylose and hexoses were absent.

In the case of none of the hemicelluloses was it possible to identify the methylpentosan constituent. Further, the very small amounts of uronic acid residues present in all cases made their absolute characterisation impossible, though, as has been shown, glycuronic acid appears to be present in at least three cases.

DISCUSSION.

It has been shown by Cunningham and Dorée [1914] and other workers, that ω-hydroxymethylfurfuraldehyde gives with phloroglucinol a precipitate which is soluble in alcohol. However, it has been shown that the hemicelluloses of the present research contain no hexosan, and it thus seems legitimate to regard the alcohol-soluble portion of the phloroglucide precipitate as due solely to methylpentosans.

From the results of the hydrolyses, and from the analytical data quoted in Table II, it is possible to calculate with fair accuracy the relative proportions of xylan, araban, methylpentosan, and uronic anhydride present in the four fractions. Use is made of the following relations:

100 g. uronic anhydride yield 16·7 g. furfuraldehyde.
100 g. xylan yield 64·5 g. furfuraldehyde.
100 g. araban yield 53·5 g. furfuraldehyde.

The calculated values are given in Table III.

Table III.

Hemicellulose	A	B 1	C 1	C 2
Total furfuraldehyde %	61·49	57·31	54·57	43·90
Furfuraldehyde from uronic anhydride %	0·63	0·87	1·23	0·93
Furfuraldehyde from pentosan %	60·86	56·44	53·34	42·97
Xylan %	94·36	87·50	82·70	—
Araban %	—	—	—	80·32
Methylpentosan %	—	7·01	7·42	8·91
Uronic anhydride %	3·76	5·20	7·40	5·56
Total percentage accounted for	98·12	99·71	97·52	94·79

Thus, it has been possible in all cases to account satisfactorily for practically 100 % of the constituents. It will be seen that hemicelluloses A, B 1 and C 1 form a series, in which increasing solubility in water is accompanied by simultaneous increases in the amounts of uronic anhydride and methylpentosan present, with corresponding decreases in the xylan percentages. However, the corresponding rotatory powers (Table II) do not show a similar progressive change.

The simultaneous occurrence in some cases of methylpentosan and uronic anhydride is of interest; this might have been expected, however, since these may be regarded as derived from hexosan by reduction and oxidation respectively.

If the above fractions represent individual hemicelluloses, it is apparent that the hemicellulose molecule is one of considerable complexity. Thus, fraction B 1, yielding 57·31 % furfuraldehyde, 7·01 % methylpentosan, and 5·20 % uronic anhydride, would correspond to a molecule of the minimum dimensions

$$(C_5H_8O_4)_{43} \cdot (C_6H_{10}O_4)_3 \cdot (C_6H_8O_6)_2$$

with a molecular weight of approximately 6500. Such a structure would give a furfuraldehyde yield of 57·47 % and would contain 6·8 % and 5·4 % of methylpentosan and uronic anhydride respectively.

The alternative view that the fractions isolated represent aggregates of molecules of similar physical properties has much to commend it, and at present it is manifestly unwise to claim that individual hemicelluloses have been isolated.

SUMMARY.

1. The hemicellulose of maize cobs has been separated into four fractions, none of which corresponds to a pure xylan.

2. Hemicellulose A yields on hydrolysis xylose and a uronic acid; B 1 and C 1 yield varying proportions of xylose, uronic acid, and methylpentose; C 2 gives arabinose, uronic acid, and methylpentose.

3. In all cases the proportion of pentose residues present far exceeds the combined proportions of residues of methylpentose and uronic acid.

The author's best thanks are due to Prof. A. R. Ling and Dr F. W. Norris for their encouragement and advice.

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CXI. CAROTENE AND VITAMIN A.

THE TRANSFORMATION OF CAROTENE INTO VITAMIN A AS SHOWN BY A STUDY OF THE ABSORPTION SPECTRA OF RAT-LIVER OILS.

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(*Received June 28th, 1930.*)

Moore [1929, 1930] has advanced evidence to show that when highly purified carotene is administered to rats it is not stored in the liver in an unchanged condition, but suffers conversion to vitamin A. Among other evidence in favour of this view, preliminary results obtained by the present author in regard to the absorption spectra of the liver oils of rats receiving carotene were quoted. These preliminary experiments have now been confirmed and the results may be described in detail.

Experimental.

The liver oils, extracted by Dr Moore, were forwarded to me in sealed tubes. After weighing, the oils were dissolved in dry ether or chloroform and their absorption spectra determined with a Hilger E 3 all-quartz spectrograph used in conjunction with a Hilger sector photometer with a condensed copper spark as light source.

The numbering of the rats corresponds with that of Moore's paper [1930]. Closely similar absorption curves were obtained for the liver oils of rats receiving similar diets and representative examples are reproduced in Fig. 1.

The results are shown in Table I.

Table I.

No. and sex of rat	Diet	Solvent	Band at 325 $\mu\mu$	Band or inflexion in region of 280 $\mu\mu$
5 ♂	Vitamin A-free	Ether	No band	Inflexion
8 ♀	"	Chloroform	"	"
9 ♂	"	"	"	"
10 ♀	"	Ether	"	"
16 ♀	Vitamin A-free + 0·75 mg. per day carotene. M.P. 178°	Ether	Good band	Shallow band
17 ♀		Chloroform	"	"
19 ♂	Vitamin A-free + red palm oil (1·5 g.)	Chloroform	"	Inflexion
20 ♀		Ether	"	Shallow band
22 ♂	Vitamin A-free + fresh carrots *ad lib.*	Chloroform	"	Shallow band

Discussion.

It is seen that in every case the liver oils of rats fed on a diet free from vitamin A gave no band in the region of 325$\mu\mu$, while the liver oils of rats given in addition either crystalline carotene or substances containing carotene showed selective absorption in this region in every case. The absorption spectra shown by the liver oils of the rats treated with carotene were therefore similar to those shown by cod-liver oils in which, as well as in other animals, vitamin A activity has been found to run parallel with a prominent band in the position stated [Takahashi *et al.*, 1925; Morton and Heilbron, 1928; Drummond and Morton, 1929]. Since it has already been shown [Capper, 1930] that this band is absent from the absorption spectrum of carotene (subsequent experiments have also shown that it is absent from the spectrum of red palm oil) it is obvious that the substance responsible (presumably vitamin A) must be synthesised *in vivo* from the carotene.

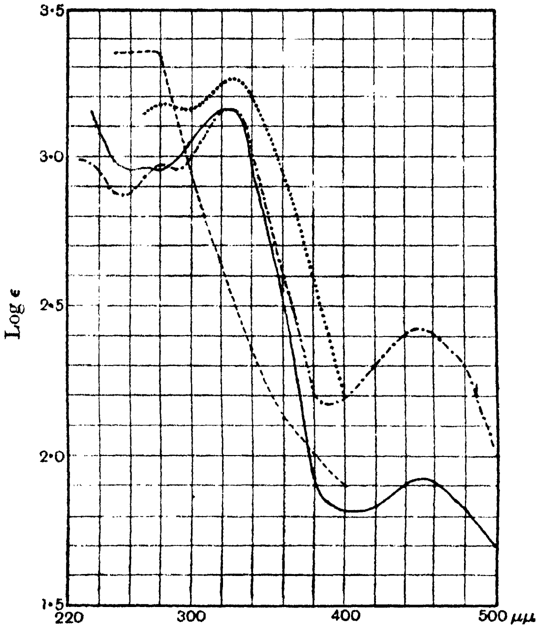

Fig. 1. - - - - - Liver oil of rat no. 10 in chloroform.
. . . . . Liver oil of rat no. 22 in chloroform.
——— Liver oil of rat no. 16 in ether.
— · — Liver oil of rat no. 20 in ether.

ε is defined by $\log I_0/I = \epsilon c d$, where c is the concentration of the liver oil in g. per cc., d is the cell thickness in centimetres.

This result not only affords support to the view that carotene is converted to vitamin A *in vivo* but in a reciprocal manner serves also to strengthen confidence in the reliability of the 325$\mu\mu$ band as an index of the presence of the vitamin. Morton and Heilbron [1928] have shown that this band is always present in sources of vitamin A of animal origin, and that when physiological activity is destroyed by oxidation the band disappears. From the present work additional evidence may be deduced, since when the vitamin, as characterised by its other attributes, is synthesised in the rat, the band simultaneously makes its appearance.

In addition to the main band at 325$\mu\mu$ shallow bands or inflexions were noted in the region of 280$\mu\mu$. Absorption in this position has been repeatedly described by various workers in the case of cod-liver oils [Schlutz and Morse, 1925; Schlutz and Ziegler, 1926; Heilbron, Kamm and Morton, 1927; Woodrow, 1928]. It has been suggested that this band may be due either to the presence of ergosterol or to a decomposition product of vitamin A, but nevertheless the actual significance remains obscure. From the present work it may be noted, without comment, that inflexions were observed even in the case of the liver oils of rats killed during acute vitamin A deficiency.

SUMMARY.

1. The band at 325$\mu\mu$ generally attributed to vitamin A was absent from the absorption spectra of the liver oils of rats suffering from vitamin A deficiency, but was shown by the liver oils of similar rats which had been subsequently cured by massive doses of carotene.

2. Since the 325$\mu\mu$ band has already been shown to be absent from the absorption spectrum of carotene, it is obvious that the substance responsible for the exhibition of this band by the liver oils of carotene-treated rats (presumably vitamin A) had been synthesised *in vivo* from the carotene.

3. Additional evidence in favour both of the conversion of carotene to vitamin A and of the reliability of the 325$\mu\mu$ band as an index of the presence of the vitamin is therefore made available.

My thanks are due to Dr Moore for his continued interest and help.

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CXII. NOTE ON THE POSSIBLE MECHANISM OF DIPHTHERIA TOXOID FORMATION.

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(Received June 30th, 1930.)

WHEN diphtheria toxin is incubated with formaldehyde the toxic properties disappear [Glenny and Sudmersen, 1921], leaving the antigenic function practically unimpaired [Ramon, 1925, 1928]. In these laboratories toxins have been prepared with an M.L.D. as low as 0·0005 cc. and Lf. of 0·024 cc. and after incubation with 0·4 % formalin the M.L.D. is considerably greater than 5 cc. whilst 90 % of the antigenic activity (as measured by the flocculating power) is retained.

The mechanism of this process is of considerable interest both from the theoretical and practical points of view since toxoid is largely used in the preparation of diphtheria antitoxin and is being employed increasingly in prophylaxis.

Formaldehyde reacts with amino-groups forming methylimino-compounds. It appears possible therefore that the toxoiding process is due to the removal of free amino-groups in the toxin which are responsible for toxicity but play no part in eliciting antibodies or combining with antitoxin.

Kissin and Bronstein [1928] claim that all free NH_2-groups in the toxin molecule are combined during the toxoiding process, but later [1930] modify this view and claim that one-half of the free NH_2-groups disappears when the toxin is treated with 0·4–0·5 % of formalin. They conclude therefore that there are two sets of amino-groups in the toxin molecule. One set is responsible for toxicity and, conveniently, disappears during the toxoiding process; and the other set, responsible for combining with antitoxin and eliciting antibodies, remains unattacked by formaldehyde.

Experiments are described in this communication to test this and other hypotheses which might explain toxoid formation.

EXPERIMENTAL.

Method of toxoid preparation.

Directions for preparing diphtheria toxoid given by various authors are as follows: the toxin is incubated at 36 to 42° with 0·3 to 0·5 % of formalin for periods ranging from 2 weeks to 2 months.

In these laboratories the following standard conditions have been found most suitable.

0·4 % of 40 % formaldehyde is added to the toxin and the mixture is incubated at 38° in a hot room for 4–6 weeks. The loss of antigenic power (as measured by flocculation methods) is of the order of 10 % only, and the toxoid produces no local or general reaction when inoculated subcutaneously in 5 cc. doses into 300 g. guinea-pigs.

To give a typical example: a 5 litre batch of toxin (M.L.D. 0·0005 cc., Lf. 0·024 cc.) was incubated at 38° with 20 cc. of 40 % formaldehyde (A.R.) for 6 weeks. 5 cc. were then inoculated subcutaneously into a 300 g. guinea-pig. No local swelling or other symptom was observed and the animal steadily gained weight. The Lf. was now 0·028 cc. Thus the toxicity had diminished at least 20,000 times, whilst 90 % of the flocculating power was retained.

The following experiment shows the effect of varying the conditions of the toxoiding process.

Original toxin, Lf. 0·09 cc., 5-litre batches used; after 4½ weeks' incubation under the conditions described, subcutaneous inoculations of 5 cc. were made into 300 g. guinea-pigs with the results shown.

Table I. *Effect of amount of formalin added on toxoid formed.*

(4½ weeks' incubation at 38°)

	Effect on guinea-pig	
Formalin added %	Change in weight after 1 week (g.)	Amount of local swelling
0·3	− 5	Large firm
0·4	+ 15	Trace
0·5	+ 25	Practically nil

Table II. *Effect of temperature of incubation on toxoid.*

(0·4 % formalin)

	Effect on guinea-pig	
Temp. ° C.	Weight after 1 week (g.)	Amount of local swelling
36	− 35	Extensive firm
38	+ 15	Trace

Table III. *Effect of* p_H *after addition of formalin.*

(0·4 % formalin at 38°)

	Effect on guinea-pig	
p_H when incubation commenced	Weight after 1 week (g.)	Amount of local swelling
8·0	± 0	Practically nil
7·3	+ 15	Trace

In another experiment one batch of toxin was incubated with 0·4 % of formalin for 6 weeks at 40–41° and another batch at 38°. In this case practically no difference was observed between the effects on guinea-pigs of toxoids prepared at 38° and 40°, but whilst the flocculating titre of that prepared at 38° had dropped some 8 %, that incubated at 40° had fallen nearly 25 %. It is inadvisable and unnecessary therefore to incubate at temperatures higher than 38°.

Amino-nitrogen of toxins.

Veal or horse-flesh infusion broth containing 2 % of Difco proteose peptone is used for toxin production. The amino-nitrogen of the medium (determined by the Van Slyke and Sørensen methods) varies between 0·065 and 0·1 %. The toxin filtrates prepared by 8–10 days' growth of Park-Williams 8 strain of *C. diphtheriae* have generally a slightly higher content of amino-nitrogen, but lower values than those of the original medium have been observed. The limits observed have been 0·06 % and 0·11 % of amino-nitrogen.

In view of Hartley and Hartley's [1922] observation of the deleterious effect of overheating the medium required for toxin production, comparisons were made of toxin production in broths sterilised by autoclaving and by candle-filtration respectively. Batches of broth were divided into two parts, both being treated in precisely the same way, save that one part was autoclaved and the other filtered through a candle to effect sterility. In each case better toxin production and higher amino-nitrogen content were observed in the batches which had not been autoclaved. No direct relationship, however, could be traced between toxin production and the amino-nitrogen content of the medium.

Table IV. *Effect of method of sterilisation of broth on properties of toxic filtrate.*

	Filtered		Autoclaved	
Batch no.	Flocculation units per 1 cc.	Amino-nitrogen %	Flocculation units per 1 cc.	Amino-nitrogen %
821	42	0·10	18	0·05
824	28	0·09	8	0·06

Action of formaldehyde on amino-compounds.

Amino-groups are determined in the Sørensen method by addition of excess of formaldehyde and titration of the acidity developed. The acid liberated is quantitatively equivalent to the amino-nitrogen combined.

Differential Sørensen titrations were performed in the following way. A small quantity of formaldehyde (approximately the theoretical quantity required to combine with all the amino-groups present) was added to the amino-compound and the acidity developed was titrated; increasing quantities of formaldehyde were then added and the acid liberated was determined after each addition.

In the following tables are given typical results obtained with a simple amino-acid (glycine), peptone, peptone infusion broth, a diphtheria toxin filtrate and a detoxified (formalinised) toxoid: 10 cc. of the neutralised solution were used in each case.

Table V. *Differential Sørensen titrations.*

40 % HCHO added (cc.)	0·1 N NaOH required to neutralise (cc.)		
	0·5 % glycine	2 % peptone	2 % peptone infusion broth
0·05	1·0	0·70	1·1
0·10	2·0	1·13	2·1
0·20	3·4	1·45	3·2
0·40	4·3	1·75	3·8
0·80	5·1	2·00	4·2
1·60	5·8	2·25	4·6
3·20	6·4	2·55	5·1

The equilibrium constant $K = \frac{[\text{glycine}]\,[\text{HCHO}]}{[\text{methylimino-compound}]}$ may be calculated from the above data. The mean value is 0·28 and divergences from this value are relatively small considering the nature of the experiments (limits 0·20 to 0·32).

Table VI.

Diphtheria toxin T. 800; Lf. 0·053 cc.		Toxoid F.T. 800; Lf. 0·056 cc.	
40 % HCHO added (cc.)	0·1 N NaOH to neutralise (cc.)	40 % HCHO added (cc.)	0·1 N NaOH to neutralise (cc.)
0·05	1·0	(0·04)*	(0)*
0·10	2·5	0·10	0·8
0·20	3·9	0·20	2·2
0·40	4·7	0·40	3·3
0·80	5·3	0·80	4·2
1·60	5·9	1·60	4·8
3·20	6·6	3·20	5·3

* Already present.

When the quantity of formaldehyde added is the amount theoretically sufficient to combine with all the amino-nitrogen present (usually about 0·05 cc. of 40 % HCHO added to 10 cc.) it is found that only some 15 % of the amino-nitrogen is combined, leaving about 85 % free. A very large excess of formaldehyde (over 6000 %) must be added before an approach to quantitative combination occurs. In the toxoiding process the amount of formaldehyde added (0·4 to 0·5 % of 40 % HCHO) is approximately the theoretical quantity to combine with all the amino-nitrogen. It is found, however, that even in the completely detoxified toxoid which has been incubated with formaldehyde for 6 weeks 85 % of the amino-nitrogen remains free and uncombined.

In Fig. 1 are plotted the results obtained with toxin T. 800 and the corresponding toxoid F.T. 800. It will be seen that the total titratable free amino-nitrogen of the toxoid is approximately 85 % of that of the toxin.

Furthermore, the same result has been obtained with every toxin and toxoid examined, and, not only so, but also with simple amino-compounds such as glycine, with peptone and with the medium in which the organisms are grown. It seems likely therefore that the same would be found true of the pure diphtheria toxin itself if it could be isolated. The same result was obtained when the Van Slyke method of determining amino-nitrogen was used in place of the Sørensen titration method. It seems justifiable to assume therefore that only about one-sixth or one-seventh of the amino-groups in toxin disappear during the toxoiding process and that toxoiding may proceed by some altogether different mechanism.

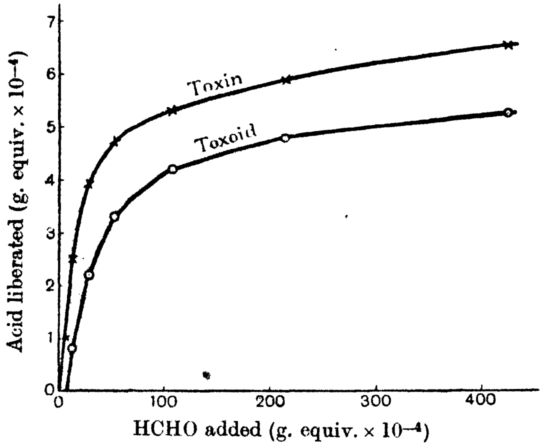

Fig. 1. Differential Sørensen titrations

This result is completely at variance with the conclusions of Kissin and Bronstein [1928, 1930] who claim that exactly 50 % of the free amino-groups in the toxin molecule disappear during the toxoiding process. Their experiments receive attention in the next section.

Acid titration curves.

Kissin and Bronstein's [1928, 1930] experiments were conducted as follows. Samples of toxin (5 cc.) were neutralised to litmus (Point I), *N* HCl was then added until the maximum precipitation was observed (Point II), then a further quantity of HCl was added until the reaction was acid to tropaeolin (Point III). If x cc. of acid were required to pass from Point I to II and y cc. to pass from II to III, the ratio x/y is known as the "acid quotient." The authors claim that the "acid quotient" of broth or of fresh diphtheria toxin is 2, whilst that of detoxified toxoid approximates to 1.

If attention is confined for the moment to the technical aspect of these experiments, a number of criticisms may be made. The use of litmus as a neutral point indicator is inadvisable owing to the wide range of colour change and the impossibility of accurate, reproducible readings. The point of maximum precipitation (II) is again not particularly well defined, there is a considerable range over which differences in precipitation are difficult to detect, and finally point III, the change of colour of tropaeolin (probably tropaeolin 00), is again susceptible to the inaccuracies involved in the observation of colour change. There is therefore considerable scope for errors in this titration method, particularly since the final "acid quotient" is the ratio of two amounts obtained by difference. A discrepancy of less than 0·1 cc. in the titrations is sufficient to render the whole experiment useless. It must be pointed out that their experiments have not been confirmed by any other method.

Before going further it was decided therefore to repeat Kissin and Bronstein's experiments. It was impossible to obtain reproducible results of any value using litmus and tropaeolin 00 as indicators so electrometric titrations with the quinhydrone electrode were employed. Varying amounts of *N* HCl were added to 5 cc. samples of the toxin under examination and the p_H was determined electrometrically. A typical pair of experimental results is illustrated in the titration curves in Fig. 2. The neutral point to litmus was assumed to be p_H 6·2 (as stated by Kissin and Bronstein), that of tropaeolin 00 to be p_H 2·0, and the points of maximum precipitation of toxins and toxoids appeared to be not widely different and at about p_H 3·7. From the curves, therefore, Kissin and Bronstein's "acid quotient" may be read off with a fair degree of accuracy. No appreciable difference between toxins and toxoids was found, in every case the "acid quotient" was between 1·3 and 1·5. Thus, when Kissin and Bronstein's experiments were performed with our toxins and toxoids under more accurate conditions their results were not confirmed.

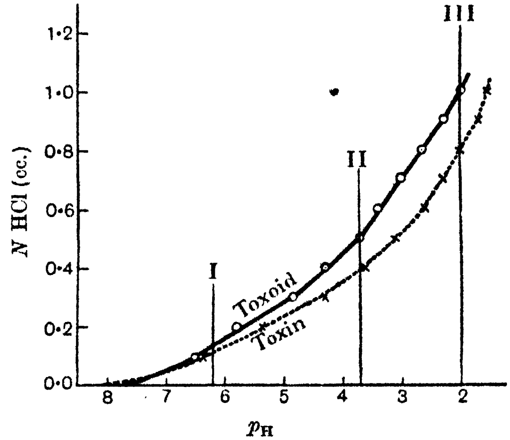

Fig. 2. Titration curves.

The only other experiment reported by Kissin and Bronstein to support their view was the determination of the amino-nitrogen of toxin by the Van Slyke method before and after the toxoiding process. A 50 % diminution in free amino-nitrogen was observed. This is completely at variance with results here reported. As mentioned in the previous section a diminution of only 15 % in free amino-nitrogen was found, both by the Van Slyke and the Sørensen methods. Furthermore, this diminution occurred directly the formaldehyde was added to the toxin and was not a gradual process like that of toxoiding. It should be remarked that 3–5 minutes' shaking at 18–20° is generally considered sufficient for the completion of the reaction of nitrous acid with amino-groups. Kissin and Bronstein allow the reaction to continue for 30 minutes—

a period likely to result in hydrolysis of peptide linkings and hence to vitiate the measurement.

The theoretical interpretations placed by Kissin and Bronstein on the results of their experiments are also susceptible to critical examination. From the determination of acid quotients they conclude that the diphtheria toxin molecule contains two free amino-groups and one carboxylic acid group and that the toxoid molecule is identical but with only one free amino-group. The tacit assumption is made that in a toxic diphtheria culture filtrate there is present nothing but pure toxin, or, at any rate, that there are present no other substances likely to interfere with the titration. Of the substances in the toxic filtrate at least 99·5 % are not toxin at all, and the buffering power of peptone, phosphates, carbonates, etc. in various ranges of p_H is considerable. It is unwarrantable, therefore, to assume that the characteristics of the titration curve are attributable to the pure toxin and that conclusions regarding the constitution of the toxin molecule may be deduced therefrom. Kissin and Bronstein state that the original broth gives the same acid quotient as diphtheria toxin filtrates. This again should make for caution in drawing conclusions from the behaviour of the very complex mixture comprised in the filtrate after growth of the culture. If the toxic filtrate cannot be distinguished from the original broth by this method, then it seems unwise to attempt any deductions from the results regarding the constitution of the toxin.

An experiment with toxin purified by acid precipitation showed a diminution of amino-nitrogen from 0·04 mg. per 1 Lf. to about 0·00016 mg., showing that not more than $\frac{1}{250}$th of the amino-nitrogen in the toxic culture filtrate was attributable to the toxin itself.

Reducing effects of formaldehyde.

The oxidation-reduction condition of a reversible oxidation-reduction system may be observed by immersing an unattackable electrode in the solution. The potential difference set up at the electrode is dependent on the proportion of oxidised and reduced forms present of the substance studied. The more highly oxidising the system, the more highly positive is the potential and the more reducing the system, the more negative is the potential.

Formaldehyde itself does not produce an oxidation-reduction potential at an electrode. By itself it is inert electrochemically but when added, for example, to fresh milk an enzyme present has an activating effect, and the mixture develops reducing properties when incubated. These reducing properties may be observed by the reduction of methylene blue (Schardinger reaction) or by measurement of the reducing potential developed [Clark, Cohen and Gibbs, 1925].

When formaldehyde was added to diphtheria toxin and the mixture was incubated a slight reducing potential was developed. For example, in one case, 0·4 % of 40 % formaldehyde was added to a sample of toxin and the mixture was incubated. The original toxin had an oxidation-reduction potential of $E_h = 0{\cdot}34$ v. but the formalinised mixture developed slowly a potential of $E_h = 0{\cdot}22$ v., at which level the potential was maintained for 10 days, the duration of the experiment.

It seemed possible therefore that the toxoiding process might be due to the maintenance of slight reducing conditions when the toxin was incubated with formaldehyde.

An alternative method of reduction was therefore attempted. It has been shown [Hewitt, 1930] that intense reducing conditions are established in diphtheria cultures during growth and that these are maintained after the cessation of active proliferation. After 9 days' incubation, therefore, a culture of *C. diphtheriae* (Park-Williams 8 strain) was sealed with melted vaselin, in order to prevent free access to air and to assist in the maintenance of reducing conditions. The incubation of the sealed culture was continued for 27 days. The filtrate of this culture was still highly toxic to guinea-pigs and the toxin-toxoid content, as measured by the Ramon flocculation method, had increased slightly during the incubation from 18 to 22 antigenic units per 1 cc. The flocculation time had also decreased slightly and the organisms proliferated actively when sub-cultured.

This method of reduction had therefore failed to produce the toxoiding process. That the toxoiding process is not due to the reducing action of formaldehyde is supported by the fact that although formaldehyde disappears during the toxoiding process the acidity corresponding to formic acid formation is not developed, as would be expected if the following formula expressed the process accurately:

$$\underset{\text{}}{H.CHO} + \underset{\text{toxin}}{T} + H_2O \longrightarrow H.COOH + \underset{\text{toxoid}}{TH_2}$$

If the formaldehyde, in its reducing effects, were oxidised still further to carbon dioxide,

$$HCHO + O_2 \longrightarrow CO_2 + H_2O,$$

there should be an increase of some 0·2 % in the CO_2 content of the toxin. Actually a decrease in CO_2 content from 0·13 % to 0·12 % was observed during the incubation with formaldehyde. During the toxoiding process a considerable proportion of the formaldehyde disappears but its transformation product has not been found.

Effect of other reagents.

Sodium hydrosulphite. To 20 cc. of toxin (T. 824, Lf. 0·04 cc.) were added 0·05 g. sodium hydrosulphite and *N* NaOH to neutrality. The mixture was incubated at 40° for 2 hours and allowed to stand in the incubator for one night. The mixture was rendered sterile by filtration through a Seitz filter and 1 cc. was inoculated subcutaneously into a 300 g. guinea-pig. The animal died in 24 hours and *post mortem* examination showed typical diphtheritic toxaemic changes. This exposure to reducing conditions had not therefore led to disappearance of toxicity.

Hydrogen peroxide. 20 cc. of toxin (T. 824) were incubated with 0·04 cc. of hydrogen peroxide (20 vol. A.R.) at 40° for 2 hours. After standing overnight on ice 1 cc. was inoculated subcutaneously into a 300 g. guinea-pig. The animal was dead in 16 hours and *post mortem* examination revealed typical acute diphtheritic toxaemia. The oxidation, therefore, had not caused disappearance of toxic effects.

Methyl alcohol. 20 cc. of toxin (T. 824) were incubated at 40° for 24 hours with 0·04 cc. of methyl alcohol (A.R.). 1 cc. was inoculated subcutaneously into a 300 g. guinea-pig which was dead in 16 hours with typical acute diphtheritic toxaemia (*post mortem* indications). Methyl alcohol, which may be produced from formaldehyde by reduction, had therefore failed to remove the toxic properties of the toxin.

CONCLUSIONS.

In the conversion of toxin into detoxified toxoid the amount of formaldehyde added is approximately the quantity theoretically sufficient to combine with all the amino-nitrogen present. Under these conditions, however, only some 15 % of the total amino-nitrogen is combined, and even after 6 weeks' incubation some 85 % of it remains uncombined. A very large excess of formaldehyde is necessary before disappearance of free amino-nitrogen approaches completion. This is found to be the case not only with toxins and with broth but with peptone and the amino-acid, glycine. It seems probable therefore that the same would be found true of pure diphtheria toxin itself if it could be isolated. Since some six-sevenths of the amino-groups in the toxin molecule probably remain uncombined when this is converted into toxoid an alternative explanation of the effect of formaldehyde seems possible.

Kissin and Bronstein [1928, 1930] maintain that 50 % of the free amino-groups in the toxin molecule disappear during the toxoiding process and conclude that the action of formaldehyde is to remove those amino-groups (50 %) which are responsible for toxic effects but not for antigenic function or combining power. It was not found possible to confirm their results, and their experimental methods and theoretical conclusions are criticised.

As an alternative to the removal of free amino-groups, another function of formaldehyde was investigated. Formaldehyde is capable of developing reducing tendencies in the presence of a suitable activator. Oxidation-reduction potential measurements indicated some activation of formaldehyde when incubated with toxin, but another method of maintaining reducing conditions, namely, that of sealing a living diphtheria culture, failed to detoxify the toxin. Formaldehyde disappears during the toxoiding process but is not converted to formic acid, or CO_2, which is again evidence that it does not function as a reducing agent in the process.

Under the conditions employed reducing and oxidising agents and methyl alcohol failed to produce a detoxified toxoid.

Possible explanations of the toxoiding effect of formaldehyde, therefore, remain as follows.

1. The disappearance of a small fraction of the free amino-groups in the toxin molecule may result in detoxification without destruction of combining power with antitoxin, or antigenic activity.

2. The formaldehyde may cause union of two or more toxin molecules with formation of a compound of high molecular weight:

$$\underset{\text{toxin}}{TNH_2} + HCHO + \underset{\text{toxin}}{TNH_2} \longrightarrow \underset{\text{toxoid}}{THN - CH_2 - NHT} + H_2O$$

The compound, having the same general configuration as the original toxin, might possibly retain antitoxin-combining power without being able to exert toxic effects.

3. The formaldehyde may react with groups other than NH_2 in the toxin molecule.

4. The formaldehyde may itself undergo transformation, such as reduction, oxidation, polymerisation or combination with other broth constituents before reacting with the toxin. In this connection two facts are of interest, firstly the slow and gradual nature of toxoid formation and secondly that hexamethylenetetramine, acetaldehyde and trioxymethylene sulphite have been found to produce toxoid formation, whilst paraldehyde does not [Berthelot and Ramon, 1925; Hollande and Penn, 1928].

Although these hypotheses are entirely speculative they may provide a working basis for further investigation.

SUMMARY.

1. Of the amino-nitrogen present in fresh diphtheria toxins some 85 % remains free in completely detoxified toxoids prepared by incubation with formaldehyde.

2. Maintenance of toxins under reducing or oxidising conditions failed to produce toxoid.

3. No evidence that toxoid formation is due to disappearance of free amino-groups, to reduction or to oxidation has been obtained. Possible mechanisms are discussed.

The author is deeply indebted to Dr R. G. White for his encouraging interest and to the laboratory staff for their cooperation.

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CXIII. THE SPECIFIC REFRACTION INCREMENTS OF SERUM-ALBUMIN AND SERUM-GLOBULIN.

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(*Received July 1st, 1930.*)

(1) Introduction.

The refractive index of a protein solution is greater than that of pure water, and, according to the observations of Reiss [1904] and Robertson [1912], the difference between the refractions of the protein solution and the solvent is equal to the protein concentration in g. per 100 cc. of solution, multiplied by a constant. This constant is termed the specific refraction increment or the specific refraction of the protein.

A critical account of the work of previous investigators on the refractions of the proteins in normal and in pathological sera has been published by Schretter [1926] who has drawn attention to the question as to whether the specific refraction increments show physiological or pathological variations. The results obtained by different observers show a wide range of variation in normal as well as in pathological sera, but further investigation is required in view of the risks of error due to the relatively low stability of the proteins and their association with lipins and other impurities.

The first problem investigated in this work is the preparation of a purified solution of crystalline horse serum-albumin under conditions which minimise the risk of error due to alterations in the protein. The purity and stability of the preparations have been tested by comparing their specific refraction increments after successive recrystallisations and after different periods of dialysis in accordance with the procedure adopted by Hopkins [1900] in establishing the individuality of egg-albumin by measurements of the optical rotation of the protein after successive recrystallisations.

The second problem is the application of Donnan's theory of membrane equilibrium to measurements of the refractive indices of serum-albumin and serum-globulin solutions which have been enclosed in collodion membranes and dialysed against phosphate mixtures of well-defined hydrogen ion concentration. An experimental investigation of the effects of the unequal distribution of salts on refraction is of value in determinations of the specific refraction increment of globulin, which cannot be dissolved in the absence of electrolytes.

The theoretical interpretation of the specific refraction increment of a protein is discussed in Section 11 in this work, and compared with determinations on amino-acids and calculations based on the refractivities of the constituent atoms.

(2) *A rapid method for the recrystallisation of horse serum-albumin.*

According to Svedberg and Sjögren [1928], serum-albumin once crystallised is a homogeneous substance of molecular weight 68,000, whereas recrystallised serum-albumin is a mixture of particles of different sizes, due to the decomposition of the protein. Their suggestion that the earlier determinations of 62,000 [Adair 1926], and 45,000 [Sørensen 1925, 1] refer to material partially decomposed will be discussed in a later communication.

In this work, an attempt has been made to determine the conditions for recrystallisation in which the protein remains unaltered. It is quite certain that the decomposition products referred to by Svedberg are not formed under all conditions, because preparations have been made in which the rotation of egg-albumin and of serum-albumin remains constant after a number of successive recrystallisations [Hopkins, 1900; Young, 1922]. This constancy of the rotation is of great importance as a proof of the stability as well as the purity of the preparations.

The methods used in this work have been tested by comparing the specific refractions of material crystallised from one to four times, instead of the specific rotations. Refractometric measurements are not quite so sensitive as the measurements of rotation, but they can be made on smaller volumes of material.

Recrystallised horse serum-albumin was prepared by a slightly modified form of the method of Hopkins. The preparations made under the conditions outlined below gave constant values for the specific refraction.

Normal sterile horse serum was mixed with an equal volume of saturated ammonium sulphate solution and stored for 1 day at 0°. The globulin precipitate was removed by centrifuging or filtration. The first crystallisation was brought about by the addition of acetic acid as described in detail by Young [1922], who recommends a preliminary washing of the serum with ether, a procedure which was followed in some of our preparations.

The solution was left to crystallise over-night at room temperature. If it is placed at 0°, amorphous matter may be deposited.

The following day the crystals were separated from the mother liquor by centrifuging, and then mixed with an approximately equal volume of a fluid prepared by adding 60 cc. of *M* sodium acetate solution and 40 cc. of *M* acetic acid to 100 cc. of a saturated solution of ammonium sulphate and again centrifuged. It is possible to wash the crystals with this fluid two or three times without much loss.

The quantities of reagents used in the process of recrystallisation given below refer to a preparation of about 2 g. of washed crystals, yielded by 100 cc.

of serum. The volume of water used to dissolve these crystals is about 50 cc. After filtration of the solution of albumin in order to remove the insoluble residue, which may consist of lipins [Young, 1922], 8 cc. of *M* sodium or ammonium acetate were added, followed by a volume of saturated ammonium sulphate solution equal to the sum of the volumes of the water and the sodium acetate (about 58 cc.). Finally, the acidity was adjusted by the addition of 10·6 cc. of a mixture composed of equal volumes of *M* acetic acid and saturated ammonium sulphate (1·33 cc. of this mixture per cc. of sodium acetate). The only reagent which need be added slowly is the mixture of acetic acid and ammonium sulphate, which may be mixed with the albumin solution in a period of about 15 minutes. Crystallisation takes place within 2 hours, and it may occur in a few minutes if the amounts of water and of ammonium sulphate are reduced, but under these conditions the crystals are very small. The solution must be stirred during the addition of acid and it is advisable to stir the solution during crystallisation.

The yield can be increased by leaving the preparation over-night at room temperature. The crystals are separated from the mother-liquor by centrifuging and redissolved and recrystallised by the same method. The yield after four crystallisations is about 1 g. per 100 cc. of serum if the process is completed in a period of 3 or 4 days, a period only slightly longer than that allotted for a single crystallisation in the experiment described by Svedberg.

The process of recrystallisation adopted in this work necessitates the preparation of more reagents than does Hopkins's original method, but it shortens the time required, because the volume of ammonium sulphate required can be calculated and added rapidly. In the original method, the ammonium sulphate must be added with caution, for a slight error of judgment in estimating the amount required may lead to the formation of amorphous precipitates.

(3) *The purification of albumin by dialysis against standard buffer solutions.*

The solutions of recrystallised albumin obtained by the method described above contained ammonium sulphate and other crystalloids which were removed by dialysis in collodion membranes. The method of dialysis adopted in this work differed from that described by previous workers, in that the solutions were subjected to a preliminary dialysis with a buffer mixture composed of sodium and potassium phosphates, before they were dialysed against distilled water, in order to convert ammonium albuminates into sodium or potassium albuminates. After the ammonia has been replaced by non-volatile bases, it is legitimate to use determinations of the ash content of the solutions as a test for the degree of purification effected by dialysis. The ash contents of solutions dialysed in different membranes for different periods are recorded in Table II, and it will be seen that the amounts of ash, which vary from 0·01 to 0·06 %, are too small to cause significant errors in refractometric measurements. For comparison with the experiments on aqueous solutions of albumin, a number of preparations at the physiological hydrogen ion concentration were made by dialysing the protein against a phosphate mixture containing 0·8/15 g.-mol. of Na_2HPO_4 and 0·2/15 g.-mol. of KH_2PO_4 per litre of solution, at 0°.

(4) *Methods for the determination of the specific refraction increment of a protein.*

The specific refraction increment, α, is calculated from measurements of refraction and of the protein concentration as defined in formula (1):

$$[R' - R_s]/C = \alpha \qquad \text{......(1).}$$

R' = the refractive index of the protein solution.

R_s = the refractive index of the solvent, at the same temperature.

C = g. of dry protein per 100 cc. of solution.

This method of defining the concentration is of significance, for if the concentration is expressed in g. per 100 g. of water or per 100 g. of solution, the specific refraction increment is not a constant, but a variable which is a function of the protein concentration.

The determinations of refraction were made by means of a Zeiss dipping-refractometer, which was immersed in the protein solution after the temperature of the fluid had become constant within 0·1°. The refraction of water was measured first, in all experiments, followed by determinations of the refraction of the outer fluid R'' and of the protein solution R'. In some experiments, the temperature of these solutions differed by 0·1° to 0·3° from the temperature of the water, and a correction was made of 0·02 refractometer scale division, or 0·0000078 in the refractive index, per 0·1°. The neglect of this correction would cause an error of 0·43 % in estimating the refraction difference $R' - R''$ for a 1 % protein solution.

In our observations on aqueous solutions of serum-albumin, it was found advisable to make readings of the refractometer for a period of at least 15 minutes. Solutions of albumin or of globulin containing salts usually gave accurate readings 1 or 2 minutes after the immersion of the refractometer in the beaker of solution. In the aqueous solutions, the refractometer readings in the first few minutes, before the boundary grows distinct, may be about 0·2 unit below the final steady values.

It should be observed that the temperature of the protein solution and of the control solution should agree within 0·1°; the absolute value of the temperature is not of great importance, for Robertson has found that the value of α is independent of temperature, over a range of 20°. Our determinations of the value of α, at temperatures between 15° and 22°, agreed within the limits of experimental error.

The following methods for the estimation of the protein concentration have been utilised in this work. For the purified solutions of serum-albumin, a known weight of the solution, contained in a platinum crucible, was dried to constant weight at 110° in a Lothar-Meyer air-oven. The weight of protein was calculated by deducting the weight of the ash. The concentration of the protein was calculated from these measurements and from measurements of the density of the protein solution.

For mixtures of albumin and phosphates this method is inaccurate, because the orthophosphates may be converted into pyrophosphates in the process of ashing. In such cases the protein concentration was calculated from Kjeldahl nitrogen determinations. The calculation depends upon an accurate determination of the percentage of nitrogen in the protein, and in view of the discrepancies of about 2 % recorded by different workers, the nitrogen content has been redetermined on specially purified material as stated in Section 5.

(5) *The nitrogen content of serum-albumin.*

The elementary analyses of serum-albumin recorded by different observers show a range of variation in the percentage of nitrogen which is considerably greater than the probable error of Kjeldahl determinations. Values which have been obtained are recorded in Table I.

Table I. *Percentage of nitrogen in serum-albumin.*

Author	% nitrogen	Method
Dumas and Cahours*	15·7–16·5	—
Brittener*	15·6	—
Stärke [1881]	16·04	Washed with alcohol and ether
Michel [1896]	15·8–16·0	Washed with alcohol and ether
Michel [1896]	15·6–15·8	Dialysed against distilled water
Maximowitsch [1901]	15·9	Dialysed against distilled water
Goldschmidt and Kahn [1929]	15·9	Washed with alcohol and ether
Piettre and Vila [1921]	15·1–15·2	Washed with acetone and ether

* Cited by Michel.

In this work, the percentage of nitrogen in horse serum-albumin was determined by the following method. Solutions of crystalline albumin were dialysed under pressure against distilled water at 0° for periods of 9 to 21 days, glass-distilled water being used in the final stages. The density of the protein solution was then measured, and a known weight of the solution was dried to constant weight in a platinum crucible in a Lothar-Meyer air-oven at 110°. After the final weighing, the protein was ashed and the weight of ash was obtained. The nitrogen content of the solution was estimated by Kjeldahl determinations on aliquot portions. The results obtained are recorded in Table II.

Table II. *Percentage of nitrogen in horse serum-albumin, purified by crystallisation and dialysis.*

Preparation no.	No. of crystallisations	No. of days dialysis against water at 0°	Protein g. per 100 cc. solution	Ash g. per 100 cc. solution	Nitrogen g. per 100 cc. protein
3·1	1	9	8·47	0·0592	15·54
3·2 *a*	1	18	7·06	0·0372	15·67
3·2 *b*	1	18	7·05	0·0391	15·51
3·3	1	18	3·11	0·0142	15·80
4·0	4	10	2·90	0·0116	15·54
4·5	4	21	3·57	0·0097	15·62
				Mean	15·60

Our results agree therefore with those obtained by Michel [1896] and by Maximowitsch [1901] on protein purified by dialysis.

As stated by Michel, further investigation is required to elucidate the cause of the discrepancy between the ratios of carbon and nitrogen in the materials purified by washing with alcohol and by dialysis. For the purpose of refractometric investigations, it seems desirable to adopt the figure obtained on dialysed protein, because the specific refraction increments calculated on the assumption that the protein concentration equals the nitrogen concentration multiplied by $\frac{100}{15 \cdot 60}$ are then comparable with determinations on aqueous solutions of albumin, in which the protein content is determined by drying the solution to constant weight.

(6) *The refractions of aqueous solutions of crystalline horse serum-albumin.*

Measurements of the density, the refraction and the concentrations of solutions of horse serum-albumin purified by different methods are summarised in Table III. The ash content of the preparations ranged from 0·06 to 0·01 %. In the preparation with the highest ash content, the correction for the refraction of the ash is less than 0·7 % of the refraction of the protein. It is not practicable to make an exact allowance for the refraction of the ash, and the true values of the corrections may be appreciably smaller than the figures given in Table III, which represent the maximum values rather than the most probable values. The ash must have contained traces of sodium and potassium phosphates, since these salts were used in order to displace ammonium compounds by substances which could be estimated at least approximately by ash analysis. The maximum correction is obtained by assuming that the ash is entirely due to the potassium and sodium phosphates, in which case the refraction of the ash equals 0·00178 times its concentration. If it is assumed that the ash is carbonate, produced by the incineration of sodium or potassium salts of the protein, the correction is reduced.

In comparing the refractions at protein concentrations from 3 to 8 % and at temperatures from 14·7° to 20·1°, it will be seen that the results confirm Robertson's conclusion that the specific refraction increment α is independent of the protein concentration.

The most important point illustrated in Table III is the agreement of the values of α for preparations subjected to different degrees of purification. Young states that the rotation of once crystallised serum-albumin is different from that of material crystallised two or three times. The once crystallised material used in this work differed from Young's preparation in that it was washed with the acetate buffer mixture referred to in Section 1, and dialysed in collodion membranes of an improved type. A certain amount of precipitation took place during dialysis, which indicates that once crystallised serum-albumin is contaminated with impurities which are insoluble when the inorganic salts are removed by dialysis in highly permeable membranes.

Since the results for material crystallised once and four times are in close agreement, it appears that under favourable conditions the stability of serum-albumin is great enough to justify recrystallisation. The agreement of the results for material dialysed for 10 to 21 days affords additional evidence in favour of the stability of the preparations.

Table III. *Refractions of aqueous solutions of horse serum-albumin.*

Reference no.	HA 3·1	HA 3·2 a	HA 3·2 b	HA 3·3	HA 4·0	HA 4·5
No. of crystallisations	1	1*	1*	1	4	4
Dialysis time (days)	9	18	18	18	10	21
Density (g. per ml.)	1·0208	1·0173	1·0173	1·0064	1·0069	1·0087
Temp. of pyknometer	20·1°	18·2°	18·2	20·1°	14·9°	14·7
Dry protein (g. per 100 cc. solution)	8·4734	7·0594	7·0547	3·1053	2·902	3·5721
Ash (g. per 100 cc. solution)	0·0592	0·0372	0·0391	0·0142	0·0117	0·0097
Temp. of refractometer	18·7°	16·7°	16·95°	20·5°	14·7°	14·5°
Refractive index of protein solution, minus refractive index of water	0·015521	0·013011	0·012989	0·005664	0·005317	0·006633
Refraction due to ash	0·000105	0·000066	0·000070	0·000025	0·000021	0·000017
Refraction due to protein	0·015416	0·012945	0·012919	0·006539	0·005296	0·006616
α (average = 0·0018295)	0·001819	0·001834	0·001831	0·001816	0·001825	0·001852
Percentage deviation (average = 0·41 %)	0·575	0·246	0·082	0·0738	0·245	1·23

* Treated with ether.

The results of previous observers are summarised in Table IV for comparison with those obtained in this work.

Table IV. *Refractions of serum-albumin obtained by different workers.*

Author	Mean value	Range	Species	Fraction
Reiss [1904]	0·00201	—	Horse	Crystalline
	0·00183	—	Horse	Non-crystalline
Robertson [1912]	0·00177	—	Ox	Total
Rohrer [1922]	0·00177	0·00173–0·00180	Human and ox	Total
Schorer [1913]	0·00188	0·00175–0·00198	Human	Total
Starlinger and Hartl [1925]	0·00166	0·00137–0·00204	Human	Total
Schretter [1926]	0·00200	0·00191–0·00212	Human	Total

The range of variation in the determinations of the specific refraction increments summarised in Table IV is considerably larger than the probable error of the measurements of the refractive index, but there are other sources of error which deserve consideration. The preparation used by Reiss was dialysed for many days at room temperature, and it may have contained the protein derivatives of low molecular weight referred to by Svedberg. The other determinations in Table IV refer to uncrystallised albumin, from pathological as well as from normal sera. The methods adopted by Robertson, Rohrer and Starlinger and Hartl have been criticised by Schretter. Schorer and Schretter calculated the protein concentration from nitrogen determinations; it is probable that their calculations are based on Stärke's determination that albumin contains 16 % of nitrogen. Guillaumin, Wahl and Laurencin [1929] adopt the figure 15·27 rather than 16. In this work the value 15·60 has been adopted. If Schretter's determination of α is recalculated on the assumption that the nitrogen content is 15·6 instead of 16 %, the result obtained is 0·00195. The experiments described below suggest that the difference between Schretter's value for total albumin and the value for the crystalline fraction obtained in this work is due partly to the existence of a non-crystalline fraction with a higher value for α and partly to impurities.

Sørensen [1925, 1] has stated that it is possible to separate crystalline serum-albumin into three fractions, characterised by differences in solubility. In one of our experiments, about half of the product was dissolved by washing with a diluted solution of ammonium sulphate, but this fractionation produced no appreciable change in specific refraction increment.

(7) *The refractions of solutions of sodium albuminate equilibrated with phosphate buffers.*

Serum-albumin in blood exists in the form of a sodium salt, and therefore it is desirable to supplement the measurements of the refraction of pure albumin with measurements of the refractions of albuminates. Preparations of albumin at the physiological hydrogen ion concentration were obtained by dialysis of the protein solutions against a standard phosphate buffer mixture, containing 0·8/15 g.-mol. of disodium phosphate plus 0·2/15 g.-mol. of potassium dihydrogen phosphate per litre of solution. The p_H value of this solution is 7·38 at room temperature and 7·41 at 0°.

The protein solutions were enclosed in osmometers and dialysis was continued at 0° until the manometers gave constant pressures. The measurements of the refractive indices of the protein solution and of the dialysate were made at about 17·5°.

The relationship between the refractive index and the concentration of the protein can be determined by the calculation of the empirical coefficient α' defined by formula 2:

$$R' - R'' = \alpha' C \qquad \text{......(2).}$$

R' = the refractive index of the protein solution.

R'' = the refractive index of the dialysate.

C = g. protein per 100 cc. solution, calculated from nitrogen determinations.

α' = an empirical coefficient, which may differ from the specific refraction increment α, on account of the unequal distribution of ions across the membrane. The mean values of α' and α are 0·00177 and 0·00183 respectively.

The results summarised in Table V and in Fig. 1 show that α' appears to have the same value for preparations from different sources and with different concentrations of the protein. Donnan's formulae, referred to below, show that α' may be increased by an increase in the protein concentration, but

Table V. *Refractions of different preparations of crystalline horse serum-albumin dialysed against a phosphate buffer* (p_H *7·4*). *In all these preparations the albumin was crystallised from solutions which had been extracted with purified ether to remove lipins.*

No.	*C* g. protein per 100 cc. solution	*α′* refraction increment	No. of crystallisations	Source
1	1·72	0·001757	1	Field labs. plasma
2	2·08	0·001760	1	,,
9	1·07	0·001798	1	Field labs. serum
8	2·47	0·001742	1	,,
7	2·49	0·001739	1	,,
6	3·54	0·001816	1	,,
5	4·86	0·001775	1	,,
19	1·48	0·001752	1	Burroughs Wellcome serum
18	1·50	0·001777	1	,, ,,
16	2·83	0·001811	1	,, ,,
17	2·99	0·001753	1	,, ,,
41	2·39	0·001755	4	Knacker serum
40	3·84	0·001770	4	,,
39	5·64	0·001759	4	,,
38	6·65	0·001769	4	,,
37	9·97	0·001791	4	,,

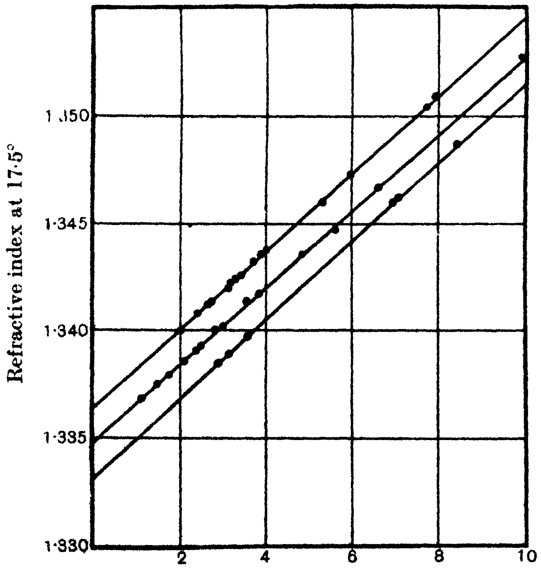

Fig. 1. Refractive indices of protein solutions at 17·5°.

Upper curve. Globulin, with 0·19 *M* KH_2PO_4 plus 0·009 *M* Na_2HPO_4.
Middle curve. Albumin, with 0·0133 *M* KH_2PO_4 plus 0·0533 *M* Na_2HPO_4.
Lower curve. Albumin dialysed against distilled water.

The lines in Fig. 1 were calculated by the following empirical formulae, in which the first constant equals the refractive index of the dialysate, and the second represents the value of α' or α.

Upper curve $R = 1{\cdot}33639 + 0{\cdot}001815C$.
Middle curve. $R = 1{\cdot}33488 + 0{\cdot}00177C$.
Lower curve $R = 1{\cdot}33320 + 0{\cdot}0018295C$.

within the range from 1 to 10 % of protein it appears that the variation is within the limits of the experimental error.

The degree of constancy in the values of α' shows that it is possible to calculate the concentration of the protein from measurements of the refractive index of the protein solution and of the dialysate by the application of formula (2). This calculation is independent of the theoretical interpretation of α'.

Schretter has pointed out that on account of the unequal distribution of ions across the membrane, it is not justifiable to assume that the refraction due to the protein can be calculated by subtracting the refractive index of the dialysate from the refractive index of the protein solution. He criticises the calculations of Rohrer [1922] which were based on the assumption that the concentration of salt is the same on both sides of the membrane. The error involved by the neglect of the salt distribution can be measured by comparing the values of α and α'. Under the conditions referred to in Table V, the difference between α and α' is about 3 %. The magnitude of the difference must depend upon the concentrations of hydrogen ions, of salts and of the protein.

The results of one experiment may be given, to show that under certain conditions, the difference α and α' must be relatively small. In this experiment, no. SA 43, a 10 % solution of sheep's serum-albumin was equilibrated with an ammonium phosphate buffer mixture at p_{H} 7·38 and 0°. The dialysate contained 0·115 g.-mol. of ammonia plus 0·0666 g.-mol. of phosphoric acid per litre. The protein solution contained 0·121 g.-mol. of ammonia plus 0·058 g.-mol. of phosphoric acid, plus 100 g. of protein per litre of solution.

The difference between the refractive indices of the dialysate and of water is equal to 0·00166 at 17·5°. The refraction of the ammonium phosphate inside the membrane must be approximately equal to 0·00166, multiplied by the distribution ratio, 0·058/0·0666, or 0·87. The total refraction due to inorganic ions must be greater than 0·0166 × 0·87, but it cannot exceed 0·00166 × 1·05, where 1·05 is the distribution ratio for ammonium ions. The difference between the refractive indices of the protein solution and of water is 0·02022, and by subtracting the maximum and minimum values for inorganic ions referred to above, it appears that the refraction due to the pure protein lies between 0·01848 and 0·01878. This conclusion was verified by a determination of α on an aqueous solution of sheep's serum-albumin, which gave the value 0·001856, or a refraction difference of 0·01856 for a 10 % solution. In this experiment, the value of α' is almost exactly equal to the value of α.

In the case of the experiments referred to in Table V, in which the distribution of ions was not determined by direct methods, approximate calculations of the concentrations of ions inside the membrane were made by a formula based on Donnan's theory.

$$S_{\mathrm{inner}} = S_{\mathrm{outer}} (1 - vx) \,.\, 10^{-\frac{1}{2}E/58\cdot 2}.$$

S_{inner} and S_{outer} denote the molar concentrations of a diffusible ion in the protein solution and in the dialysate, x, the number of g. protein per cc. of solution, v is equal to the effective volume of 1 g. of the protein (according to Weber and Nachmannson [1929] $v = 1$ cc)., $n =$ the valency of the ion, and E is the membrane potential in millivolts at 0°. In the case of a 1 % solution of serum-albumin, the membrane potential is about 0·32 millivolt. The degree of accuracy of the formula has been investigated in a previous paper [Adair, 1928].

An approximate statement of the relationship between α' and α can be obtained by applying Donnan's equations for the distribution of ions across membranes, supplemented by the assumptions formulated in equation (3).

$$R' - R'' = (\alpha + \beta)\, C + \alpha_{\text{K}}\, (\text{K}_{\text{inner}} - \text{K}_{\text{outer}}) + \alpha_{\text{Na}}\, (\text{Na}_{\text{inner}} - \text{Na}_{\text{outer}}) \ldots (3).$$

$C =$ g. pure protein per 100 cc. of solution.

β is a factor approximately equal to 0·00003, which represents the effect of the sodium or potassium combined with the protein at p_{H} 7·4 (approximately 0·00043 equivalents per g. protein). The factor β was determined by comparing the refractions of aqueous solutions of the pure protein and of sodium proteinates.

α_{K} is an empirical coefficient, $=$ 0·016, defined by a formula resembling equation 1.

$\text{K}_{\text{inner}} =$ the concentration of KH_2PO_4 in the protein solution in g.-mols. per litre of solution.

$\text{K}_{\text{outer}} =$ the concentration of KH_2PO_4 in g.-mols. in the dialysate.

α_{Na} is an empirical coefficient $=$ 0·0279.

Na_{inner} and Na_{outer} refer to the concentrations of Na_2HPO_4. In the dialysate the molar concentrations of KH_2PO_4 and Na_2HPO_4 are equal to 0·01333 and 0·05333 respectively. In the protein solution their concentrations are a function of the protein concentration. In the case of a 1 % solution ($C = 1{\cdot}0$), the concentrations of KH_2PO_4 and Na_2HPO_4 are approximately equal to 0·01303 and 0·05142 respectively. These figures are provisional estimates based on the application of Donnan's formulae.

The value of $R' - R''$ is equal to 0·0177 in the case of a 1 % solution, and by applying formula (3) to the experimental data given above, it is possible to calculate the value of α. The result obtained, 0·00180, is in fairly close agreement with the more accurate value 0·00183 obtained by direct measurement. This agreement is of significance in the determination of the specific refraction increment of globulin, in which it is necessary to rely upon indirect methods for the determination of the specific refraction increment.

(8) *The effects of purification processes on the refraction of sodium albuminates.*

In the previous section it has been shown that the refraction increment α', defined by formula (2), is a constant in the case of solutions of albumin which have been purified by washing with ether. The measurements on material which has not been purified by ether, recorded in Table VI, show a wider range of variation. The mean value of α' for the once crystallised albumin is 0·00183. The mean for twice crystallised and four times crystallised albumin is 0·00180. Hopkins [1900] and Young [1922] have observed that crystals of serum-albumin are associated with lipins, and it is not improbable that the value 0·00183, which exceeds by 3·3 % the refraction of the protein purified by ether, is due to the association of the protein with traces of lipins. The observations on protein crystallised four times agree better among themselves, and the average value 0·00180 is closer to 0·00177, the result for material treated with ether. Theoretically, the use of ether may be open to objection on the ground that it may alter the properties of the protein, but the results recorded above indicate that its use is justifiable.

Table VI. *Refractions of different preparations of crystalline horse serum-albumin dialysed against phosphate buffer at* p_H *7·4. The preparations differ from those in Table V in that the solutions were not treated with ether.*

No.	C g. protein per 100 cc. solution	α' refraction increment	No. of crystallisations	Source
13	1·94	0·00184	1	Burroughs Wellcome serum
12	2·19	0·00184	1	,, ,,
15 *a*	3·66	0·001842	1	,, ,,
14	4·64	0·001775	1	,, ,,
21 *a*	1·96	0·00184	1	,, ,,
22 *a*	1·97	0·00179	1	,, ,,
20	3·62	0·001816	1	,, ,,
35	2·44	0·001821	1	,, ,,
34	4·61	0·001823	1	,, ,,
33	6·60	0·001872	1	,, ,,
36	7·41	0·001843	1	,, ,,
32	9·14	0·001845	1	,, ,,
23	2·05	0·00177	2	,, ,,
24	1·86	0·001795	4	,, ,,
25	1·96	0·001812	4	,, ,,
27	3·81	0·001804	4	,, ,,
26	3·87	0·001813	4	,, ,,
28	1·58	0·001812	4	,, ,,
29	1·60	0·001820	4	,, ,,
30	2·82	0·001784	4	,, ,,
31	3·13	0·001809	4	,, ,,

(9) *The refraction of uncrystallised albumins from horse serum and sheep serum.*

The mother-liquor from two preparations of albumin crystals was dialysed against the phosphate buffer mixture defined in section (3). The value of α' for the first preparation was 0·001821. In the second preparation, the mean value of α' was 0·00188, a figure which is 6·2 % greater than the refraction increment of the crystallised albumin. Since a part of the protein in the mother-liquor must be crystalline albumin remaining in solution, it may be inferred that the mother-liquor contains a protein with a refraction increment greater than 0·00188.

One experiment was made on albumin from sheep's plasma. The globulin was removed by half-saturation with ammonium sulphate and acetic acid was added. The albumin formed an amorphous precipitate instead of the crystals which appear when horse serum is used. The precipitate was redissolved and reprecipitated and then dialysed against distilled water. The specific refraction increment α, defined by formula (1), was 0·00185. The protein concentration in this experiment was calculated from nitrogen determinations. Within the limits of experimental error, this agrees with the results for crystalline horse serum-albumin recorded in Table IV.

Sheep serum, like horse serum, may contain substances with a higher refraction than the protein crystallised or precipitated by acid, for the total albumin in one preparation gave a value of 0·00194 for α', the refraction against a phosphate mixture. This preparation was not treated with ether, and therefore an appreciable fraction of the refraction increment may be due to lipins.

(10) *The refractions of isoelectric globulins and of sodium globulinates equilibrated with phosphate buffers.*

Direct determinations of the specific refraction increment for globulin have been made by Robertson and other workers, who have precipitated globulin by dilution or dialysis and redissolved the precipitated globulin by the addition of electrolytes. Robertson worked with very dilute solutions, as the opalescence of his preparations made it impossible to determine the refractive indices in concentrated solutions.

Preliminary experiments made in this work indicated that clear solutions of globulin could be obtained from material precipitated by ammonium sulphate and purified by dialysis against standard phosphate mixtures. The coefficient α' can be determined directly by measuring the refractive indices of the protein solution and the dialysate, and the value of α can then be calculated by formula (3), which allows for the effects of the unequal distribution of ions.

Svedberg and Sjögren [1928] state that globulin is an unstable substance, rapidly decomposed during the processes of fractionation into "euglobulin" and "pseudoglobulin." In order to minimise the risk of error due to decomposition of the protein, a rapid method of preparation was adopted in this work. The precipitate of globulin obtained by the addition of an equal volume of saturated ammonium sulphate to normal horse serum was separated by centrifuging. It was purified by the addition of water and reprecipitation with ammonium sulphate not more than three or four times, then cooled to 0° and dialysed in collodion membranes against standard phosphate mixtures. The protein appears to be fairly stable at 0°, because the values of α' for different preparations dialysed for different periods are in close agreement.

Table VII. *Nitrogen content of horse serum-globulin.*

No.	C g. dry protein per 100 cc.	g. nitrogen per 100 g. dry protein	Difference. Refractive index protein solution minus dialysate	α' calculated from dry weight determinations
G 7	3·162	15·24, 15·19	0·00582	0·00184
G 16	12·069	15·05, 15·06, 15·00	0·02219	0·00184
G 17	7·96	15·07, 15·06, 15·06	0·01452	0·00183
G 18	5·94	15 22, 15·24	0·01086	0·00183
G 21	10·421	15·16, 15 18	0·01909	0·00183

In five experiments, given in Table VII, the protein concentration C in g. dry protein per 100 cc. of solution was determined by the method of Devoto, as described by Hopkins and by Young. The removal of the last traces of sulphate from the heat-coagulated protein may require 10 days' washing in distilled water. For most of the time the material was kept at 0°. The nitrogen content of these solutions was determined by Kjeldahl's method and the percentages of nitrogen in the dry protein are given in Table VII. The mean value obtained for total globulin is 15·13 g. nitrogen per 100 g. dry protein.

Table VII gives the values of α' calculated from the dry weight determinations and measurements of refractive indices of solutions of globulins equilibrated with a mixture containing 0·19 g.-mol. of KH_2PO_4 plus 0·009 g.-mol. of Na_2HPO_4 per litre of solution. The p_H of this solution, 5·35, is in the isoelectric region.

Table VIII. *The refractions of globulin from horse serum equilibrated with phosphate buffer* p_H *5·35.*

No.	C	α'	No.	C	α'
1	3·30	0·001813	11	7·74	0·001817
2	3·39	0·001803	16	12·00	0·001848
3	4·02	0·001828	17	7·93	0·001832
6	5·33	0·001796	18	5·98	0·001817
7	16·98	0·001827	19	3·92	0·001825
8	15·91	0·001826	20	4·01	0·001805
9	11·92	0·001762	21	10·45	0·001828
10	9·84	0·001797	22	2·43	0·001813

In the experiments summarised in Table VIII the values of α' have been calculated from nitrogen determinations.

The mean value of α' for the isoelectric protein is equal to 0·001815. According to Donnan's theory, the concentration of the diffusible ions should be the same on both sides of the membrane at the isoelectric point. It is, however, necessary to make a correction for the volume of the protein. In the case of an ideal solution of an isoelectric protein, the correction can be made by the following formula:

$$\frac{R' - R''}{C} = \alpha' = \alpha - 0{\cdot}01 v_1 (R'' - R_{H_2O}).$$

v_1 = the volume occupied by 1 g. protein, plus the volume of its water of hydration. According to Weber and Nachmannson [1929], the effective volume of 1 g. globulin is 1·3 cc.

R'' = 1·33639 at 17·5°.

R_{H_2O} = 1·33320 at 17·5°.

In a 1 % solution of the protein the concentration of the diffusible salts is diminished by 1·3 %. By applying the formula given above, it appears that $\alpha = \alpha' + 0{\cdot}000041 = 0{\cdot}001857$.

In addition to the measurements of the isoelectric point, observations have been made on globulin equilibrated with the phosphate mixture used in the work on albumin, as stated in Table IX.

The mean value of α' for this medium is 0·00181. If it is assumed that the difference between α and α' for globulin is the same as that observed for albumin (0·00006) it follows that $\alpha = 0{\cdot}00187$.

Table IX. *The specific refraction increments of globulin from horse serum and sheep serum, equilibrated with phosphate buffer mixture.* $\frac{0{\cdot}8}{15}$ *M* Na_2HPO_4 *plus* $\frac{0{\cdot}2}{15}$ *M* KH_2PO_4. p_H *7·4.*

No.	g. protein per 100 cc. solution	α'	Species	Notes
100	7·80	0·00178	Sheep	—
101	14·00	0·00180	Sheep	—
G 4	2·75	0·00182	Horse	Serum treated with ether
G 5	2·72	0·00181	Horse	Serum treated with ether
G 24	1·64	0·00181	Horse	Stored 8 months

Table X. *Determinations of the specific refraction increment of globulin.*

Author	Range	Mean	Mean re-calculated	Species
Reiss	—	0·0023	—	Horse
Robertson	—	0·00229	—	Ox
Rohrer	0·00172–0·00180	0·00177	—	Human and ox
Schorer	0·0023 –0·0027	0·00245	0·00232*	Human
Starlinger and Hartl	0·00214–0·00312	0·00265	—	Human
Schretter	0·00206–0·00234	0·00219	0·00208*	Human
Adair and Robinson	0·00180–0·00189	0·00186	—	Horse

* These recalculations have been made because it is probable that Schorer and Schretter used Hammarsten's estimate that globulin contains 16 % of nitrogen in calculating the protein concentrations from Kjeldahl determinations. The value found in this work is 15·13 %.

(11) *The theoretical significance of the specific refraction increment of a protein.*

An approximate statement can be made of the theoretical correlation of the specific refraction increment and the refractive index of the pure protein. The refractive index has been computed by Robertson [1918], whose formula is based on the assumption that Gladstone's formula [Gladstone and Dale, 1863] for the refractions of mixtures is applicable to protein solutions, and that the specific volume of a dilute protein solution is equal to unity.

Gladstone's formula is

$$(R-1)\,v = (R_p-1)\,v_p x + (R_s-1)\,v_s(1-x).$$

R, R_p and R_s denote the refractive indices of the solution, the pure protein and the solvent.

v, v_p and v_s denote the specific volumes of the solution, the protein, and the pure solvent.

x = g. of protein per g. of solution.

Robertson's assumption that $v = 1\cdot0$ leads to the conclusion that

$$\alpha = 0\cdot01\,(R_p v_p - v_p - R_s + 1).$$

A more accurate formula, numbered (4) below, can be obtained by assuming that the specific volume of the solution is equal to $xv_p + (1 - x)\,v_s$. Gladstone's formula is then equivalent to the following simple expression:

$$R - R_s = 0\cdot01 C v_p\,(R_p - R_s) \qquad \text{......(4).}$$

C = g. solute per 100 cc. of solution $= x \times 100/v$.

If it is assumed that the solute is combined with g g. of water per g. of dry material, the formula requires modification

$$R - R_s = 0\cdot01 c\,(1 + g)\,v_p'\,(R_p' - R_s) \qquad \text{......(5).}$$

R_p' = refractive index of hydrated solute of specific volume v_p'.

C = g. anhydrous solute per 100 cc. of solution.

According to the data of Sørensen [1925, 2], $g = 0\cdot303$ for serum-albumin and 0·343 for serum-globulin.

By applying formula (5) to the values of α for albumin and globulin, 0·00183 and 0·00186 respectively, it appears that the refractive indices of the pure protein hydrates are approximately 1·514 and 1·511. These figures are approximations, because it is not yet possible to make allowances for solution-contraction volumes and for alterations in the refractivity of water [Fajans, 1927]. In the case of inorganic salts, the refractive index calculated from observations on solutions may differ by more than 3 % from the refractive index of the crystalline solid.

It is possible that the water of hydration in the protein crystals differs according to the composition of the mother-liquor, and the refractive indices of the crystals should show corresponding variations.

(12) *The specific refractivity of amino-acids and proteins.*

Gladstone and Dale define the specific refractivity of a substance by the formula $(R_p - 1)\,v_p$. An alternative definition, based on the formula of Lorentz and Lorenz, has been used by Brühl [1891], but the simpler equation of Gladstone and Dale is sufficient for the purpose of this work. The materials required for the calculations of the specific refractions of certain amino-acids are given in Table XI. The values of α have been calculated for comparison with the values of α given for the proteins. It was observed that the amino-acids differ from the proteins in that α is not a constant, but tends to increase as the degree of dilution is increased. A similar increase takes place

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2. The purity and stability of the prepara[illegible] ments of their refractive indices, and it has been found that the specific refraction increment is a constant, unaffected by recrystallisation. In aqueous solutions the specific refraction increment of crystalline serum-albumin is 0·00183.

3. The refractive indices of solutions of alkali albuminates, equilibrated with phosphate buffers, have been measured and represented by a simple empirical formula, which can be used for calculations of the protein concentration from refractometric measurements. A calculation of the specific refraction increment of the protein from measurements of the refractive indices of the solution and the dialysate has been made by the application of Donnan's theory of membrane equilibrium.

4. The specific refraction increments of the uncrystallised fractions of albumin are larger than 0·00183.

5. The preparation of clear solutions of horse serum-globulin is described. The nitrogen content of total globulin has been redetermined as 15·13 %. The refractive indices of solutions of isoelectric and of alkaline globulins equilibrated with phosphate buffers have been measured and the specific refraction increment of the protein calculated, after making corrections for the effects due to the unequal distribution of ions. The mean value obtained for total globulin is 0·00186.

6. Preliminary determinations indicate that the specific refraction increments of both albumin and globulin prepared from sheep's serum are approximately equal to 0·00185.

7. Measurements have been made of the refractive indices of solutions of four amino-acids, namely, glycine, alanine, valine and tryptophan.

8. The theoretical significance of the specific refraction increment of a protein is discussed.

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